Cytokine and Antibody Responses of Allergic and Non-Allergic Subjects to Cat Antigen and Influences of IL-15 on Cytokine Production in Recall Responses

by:

Shirley Clarice Fitzpatrick-Wong

A Thesis Submitted to the Faculty of Graduate Studies
in Partial Fulfilment of the Requirements for the Degree of:

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CYTOKINE AND ANTIBODY RESPONSES OF ALLERGIC AND NON-ALLERGIC SUBJECTS TO CAT ANTIGEN AND AND INFLUENCES OF IL-15 ON CYTOKINE PRODUCTION IN RECALL RESPONSES

BY

SHIRLEY CLARICE FITZPATRICK-WONG

A Thesis/Practicum submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

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☺ live long and prosper ☺

ABSTRACT

The cytokine responses of fresh human cells to environmental allergens, such as cat antigen are not well characterized, because many previous studies have used long term clones or stimulated fresh peripheral blood mononuclear cells (PBMC) with polyclonal activators. The response is believed to be Th2-dominated. IL-4, a Th2 cytokine, causes B cell switching to IgE, the major effector antibody in allergy. We stimulated human PBMC of allergic and non-allergic subjects with cat antigen *in vitro* to determine whether Th2-like responses were associated with IgE, measured by ELISA *ex vivo* and clinical sensitivity.

We have shown that allergic subjects have increased clinical sensitivity, elevated specific and total IgE, possibly lower specific IgG4, and produce a more Th2-like response to cat antigen challenge *in vitro* (higher IL-4 and lower IFNγ). The normal subjects do respond to cat antigen stimulation with cytokine production and specific antibodies, including IgE, however, they do not display clinical sensitivity. The antigen specificity of the stimulation was confirmed by independent tests with chloroquine and anti MHC Class II antibodies. Interestingly, anti MHC Class I antibodies also blocked antigen specific cytokine responses. The population as a whole displayed a positive correlation between antigen-driven IL-4 and specific IgE, and a negative correlation between IFNγ and specific IgE, arguing that Th2-like responses are associated with specific IgE production. The specific IgE correlated to the wheal intensity and therefore we have concluded that antigen-driven cytokine production, antigen-specific IgE and clinical sensitivity are directly related.

IL-15, a novel cytokine, has been found to exhibit many functions similar to IL-2 *in vitro*, and shares some receptor subunits with IL-2. However, differences in cellular and tissue expression of the cytokines and their receptors has led to speculation that they have different functions *in vivo*. We recently described IL-2 as a Th1-promoting cytokine and now have tested the effects of exogenous IL-15 on cytokine production by human PBMC cultures. We have found that IL-15 does not elicit non-specific IFNγ or IL-10, nor augment antigen stimulated IL-10 or IL-5, but does increase antigen stimulated IFNγ. We conclude that IL-15 supports or promotes Th1-like responses.

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ABBREVIATIONS

Alternaria alternata	Alt a
Ambrosia artemisifolia	Amb a
Antigen	Ag
Antigen presenting cell	APC
B lymphocytes	B cells
Bacillus Calmette Guérin	BCG
Biological allergy units	BAU
Bovine serum albumin	BSA
Canis familiaris	Can f
Cytotoxic T lymphocyte	CTL
Daltons	Da
Dermatophagoides pteronyssinus	Der p
Dulbecco's minimum essential medium	DMEM
Enzyme linked immunosorbent assay	ELISA
Ethylene diamine tetra acetic acid	EDTA
Felis domesticus	Fel d
Fetal calf serum	FCS
Granulocyte macrophage colony stimulating factor	GM-CSF
Human immunodeficiency virus	HIV
Human leukocyte antigen	HLA
Hypoxanthine, aminopterin, thymidine	HAT

Immunoglobulin (M,D,E,G,A)	Ig(M,D,E,G,A)
Interferon	IFN
Interleukin-(1 to 15)	IL-(1 to 15)
Interleukin-(1 to 15) receptor	IL-(1 to 15)R
Lipopolysaccharide	LPS
Lolium perenne	Lol p
Lymphokine activated killer cells	LAK
Major histocompatibility antigens	MHC
Messenger ribonucleic acid	mRNA
Microgram	µg
Milligram	mg
Milliliter	ml
(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium	MTS
Nanogram	ng
Natural killer cells	NK cells
Non-idet P-40	NP40
Osteoarthritis	OA
Ovalbumin	OVA
Penicillin G sodium, streptomycin sulfate, amphotericin B	PSF
Peripheral blood mononuclear cells	PBMC
Phenazine methosulfate	PMS
Phleum pratense	Phl p

Phorbol 12-myristate,13-acetate	PMA
Phosphate buffered saline	PBS
Phytohemagglutinin	PHA
Picogram	pg
Radioallergosorbent test	RAST
Recombinant interleukin	rIL-
Rheumatoid arthritis	RA
Roswell Park Memorial Institute	RPMI
T lymphocytes	T cells
Transforming growth factor	TGF
Tumor derived activated cells	TDAC
Tumor necrosis factor	TNF
Units	U
X linked severe combined immunodeficiency	X-SCID

INTRODUCTION

ALLERGY

Allergy is an undesirable inflammatory response specific to an allergen (foreign antigen causing allergic response), which manifests itself clinically as allergic rhinitis, atopic dermatitis, anaphylactic shock, allergic asthma, or food allergy in a subset of individuals. It affects up to 20-25% of the population in developed nations. Allergens can be tree, grass or weed pollens, foods, insect venoms, house dust mites, latex, animal danders or a wide variety of seemingly innocuous environmental antigens. Some allergens are constitutive in our environment while others are seasonal.

Upon exposure to an allergen, individuals genetically predisposed to allergy produce IgE antibodies specific for the allergen. The IgE binds to the high affinity IgE receptor, $Fc_{\epsilon}RI$, on mast cells and basophils. When the individual is exposed to the allergen for a second and all subsequent times, the bivalent or multivalent allergen can bind to more than one mast cell bound IgE molecule and cause cross-linking of the receptors. The cross linking of the receptors sends a signal to the mast cell to degranulate and release mediators. Histamine is one of the most important immediate mediators. The effects of histamine are increased vascular permeability, smooth muscle contraction and mucus production. The cross linking of receptors also triggers the arachidonic acid cascade. The arachidonic acid metabolites are prostaglandins via the cyclooxygenase pathway and leukotrienes via the lipoxygenase pathway, both of which like histamine, are pro-inflammatory. The triggered mast cell also releases IL-4.

IgE is a minor isotype (class) of immunoglobulin. It is usually present in the blood as $< 0.01\%$ of total immunoglobulin. It is secreted by B cells which have differentiated

into plasma secreting cells. It does not have the hinge region which most antibody classes do and it is heat labile at a lower temperature (56°C) than other antibody classes. IgE is believed to be important in the immune response to parasites, because the Fc (constant) portion of the antibody binds to eosinophils which have cytotoxic activity. In allergy, IgE for non-pathogenic foreign antigens binds to mast cells which have inflammatory potential.

Mast cells can be placed in two categories; those present in connective tissues and those present in mucosal tissue. In mouse they have different requirements, stem cell factor for connective tissue mast cells and IL-3 for mucosal mast cells. In human they differ in the proteases they express; tryptase for mucosal mast cells and both tryptase and chymase for connective mast cells. Basophils, in contrast, are present in circulation. The immunoglobulin receptors found on mast cells include the high affinity IgE receptor $Fc_{\epsilon}R1$ which binds IgE with high affinity, and the $Fc_{\gamma}R$ which binds IgG. Mast cell degranulation due to cross linking of numerous $Fc_{\epsilon}R1$ is not infinitely linearly related to the amount of cross linking. That is, the optimal level of cross linking is not the maximal level of cross linking. Studies measuring mast cell histamine release (as a measure of mast cell activation) in the presence of anti IgE described a normal distribution of histamine release as cross linking increased.

Treatment of Allergy

The best treatment for atopic disorders is avoidance of the allergen, however, with many allergens this is not feasible. There are pharmaceutical treatments which aim to prevent or alleviate the symptoms of allergy. For example, antihistamines are of some value because they are H1 receptor antagonists, and β-adrenergic receptors or

glucocorticoids inhibit the induction of inflammation.

Immunotherapy treatment, in contrast, aims to stop allergy itself. Subjects undergoing immunotherapy treatment receive increasing doses of the allergen (for safety reasons initial doses are very low) for a period of several months or years with the goal of reducing IgE levels and, ultimately, clinical sensitivity. The effectiveness of this treatment is questionable, because many studies have not been properly controlled and the effects are frequently temporary and variable. Other isotypes may increase or decrease. The mechanism by which immunotherapy may suppress allergy is unknown but was thought to be (i) induction of "blocking" IgG antibodies or (ii) activation of suppressor T cells. It is now more commonly believed to be downregulation of T cell activity possibly due to anergy, but definitive evidence for the mechanism of successful allergen immunotherapy remains to be obtained. Recent studies have shown that clinical improvement after conventional allergen immunotherapy is associated with decreased T cell proliferation to allergen (Greenstein 1992). Indeed, a study of ten bee venom allergic subjects undergoing immunotherapy demonstrated decreased allergen-driven proliferation of PBMC during the course of treatment (Jutel 1995). They also showed a shift in the cytokine responses. PBMC cultured seven days with allergen followed by polyclonal stimulation with OKT3 $F(ab)_2$ monoclonal antibody (specific for CD3 of the T cell receptor) produced significantly decreased IL-4 and IL-5 levels and increased those of IFNγ after immunotherapy. The control for this study was that PBMC cultured with tetanus toxoid (an irrelevant antigen) did not significantly alter cytokine production as immunotherapy progressed.

Peptide immunotherapy is a newer type of treatment which aims to stimulate and eventually tolerize/anergize T cells (reviewed by Norman 1993; Wallner 1994). These small T cell peptides do not react with IgE (or other antibodies) and therefore are safer and they would be easier to standardize. The lack of reactivity with IgE would allow markedly higher amounts of allergen (peptide) to be safely administered, thereby (putatively) increasing the speed and efficacy of therapy. Studies with fresh human cells *in vitro* and *in vivo* are required, and must examine the extent and conditions of T cell unresponsiveness and whether this state can be maintained long term. Altered peptide ligands have been suggested as more effective for this type of treatment. These suboptimal ligands may lead to weaker or qualitatively different signals to the T cell and result in partial activation (reviewed in Kersh and Allen 1996). How to objectively measure efficacy of any immunotherapy treatment can be difficult. Symptom scores are very subjective. Clinical improvement is not always associated with lower IgG and IgE antibody levels. Perhaps T cell proliferative or cytokine responses will be more useful. This remains to be determined.

Cat Allergy

Cat allergy and cat-induced asthma have been shown to affect 2-10 % of the U.S. population (Gergen 1987), and cat antigen is difficult to avoid (veterinary services market 1983) or to clean away (Klucka 1995). The major cat allergen is called *Felis domesticus* 1 (Fel d 1). The only other characterized cat allergen is cat albumin. Fel d 1 is a stable 39 kDa heterodimer composed of two monomers of 18 kDa. Each monomer can be reduced and alkylated to produce two chains, one of 14 kDa and one of 5 kDa. Both chains have

been shown to elicit T and B cell responses (Rogers 1993). Over 80% of cat allergic subjects respond with IgE to Fel d 1. The *in vivo* importance of this major allergen was confirmed when it was found that monospecific anti Fel d 1 antibodies could block cat antigen-induced histamine release from basophils of cat allergic subjects (Kleine-Tebbe 1993).

For diagnosis of cat allergy and for research purposes both natural, purified extracts containing Fel d 1 and recombinant Fel d 1 have been used. The advantage of recombinant allergens is their purity and low risk of containing immunogenic contaminants. This feature would be particularly advantageous in an immunotherapeutic setting. However, the disadvantages are that they are not glycosylated like the natural product (which may or may not be relevant) and they do not contain the minor allergenic components. In our studies we have chosen to make use of a purified standardized natural cat hair extract. In this and other aspects of the study we have consciously chosen the experimental model most natural, most similar to the *in vivo* condition possible.

$CD4^+$ T CELL PHENOTYPE

T cells can be divided into two types; the $CD8^+$T cells and the $CD4^+$T cells. $CD8^+$ T cells recognize peptides in the context of MHC Class I molecules and display cytotoxic activity. In contrast, $CD4^+$T cells recognize peptides in the context of MHC Class II molecules and display 'helper' activity through the release of cytokines. Endogenous peptides are presented by MHC Class I molecules, whereas exogenous peptides are presented by MHC Class II molecules. Therefore the source of the antigen determines the

type of response elicited. Allergens being exogenous proteins are presented to $CD4^+$T by MHC Class II molecules.

Cytokines are pleiotropic immunoregulatory molecules produced by diverse cells. They are present in low concentrations, have short half lives and act across the immunological synapse in a paracrine or autocrine manner.

It was shown that long term murine $CD4^+$ T cell clones produced one of two subsets of cytokines (Mosmann 1986). The clones producing the subset which included IL-2, IFNγ, TNFβ, TNFα, IL-3, and GM-CSF were named T helper 1 (Th1), while the clones producing the subset which included IL-4, IL-5, IL-6, IL-10, IL-13, TNFα, IL-3, and GM-CSF were named T helper 2 (Th2). The Th1 response enhances microbicidal activity of macrophages and cell mediated immunity including cytotoxic T cell activity making it an appropriate response to intracellular microbial infections. The Th2 response enhances humoral immunity in part by stimulating B cells to differentiate into Ig secreting plasma cells which makes it an appropriate response to nematodes, parasites and microbes with extracellular habitats.

Human Th1 and Th2 clones were also identified (reviewed in Romagnani 1991). Human IL-10 has not clearly been defined as a Th1 or Th2 cytokine in the human system as it was in the murine system. Del Prete et al. demonstrated that human IL-10 is produced by both Th1 and Th2 clones and in contrast to murine IL-10, human IL-10 inhibits the proliferation and lymphokine production of Th1 and Th2 cells (Del Prete 1993). The relationship between the cytokine production patterns observed in long-term human and murine clones versus fresh, normal T cell populations remains to be seen.

There is cross-regulation between cytokines of different subsets. For instance IL-4 and IL-10 of the murine Th2 group inhibit the microbicidal effects of IFNγ, and IL-4 also acts directly on T cells to inhibit development of IFNγ producing cells. IL-10 diminishes IL-2 and IFNγ production by primed T cells indirectly through its actions on macrophages. IFNγ of the Th1 group inhibits the development and proliferation of IL-4 producing cells at suboptimal levels of IL-4. This is thought to be mediated through the IFNγR. One of the IFNγR chains, the IFNγRβ chain, is expressed by Th2, but not Th1 murine clones and mediates suppression through differential gene expression in the Th2 cells (Pernis 1995). Previous to this finding there had been no identified surface markers for Th1 and Th2.

It is in debate whether Th1 and Th2 cells arise from a common less mature precursor (Th0) or from lineage specific precursors. The Th0 theory states that factors present early in activation, principally cytokines, determine the differentiation outcome of the Th0 to a Th1 or Th2. Evidence for the Th0 theory comes from studies in which purified naive T cells from transgenic mice were cultured with antigen and APC as well as exogenous cytokines and subsequent cytokine production was measured. If cultured without exogenous cytokine the cells produced IL-2 but no IL-4 or IFNγ, but if IL-4 was added to culture the cells produced IL-4 (Seder 1992; Hsieh 1992). If IL-12 was added to culture, the cells produced IFNγ (Seder 1993; Hsieh 1993). The conclusions of these studies was that IL-4 priming is required by the Th0 to differentiate to Th2. The IL-4 source *in vivo* could be mast cells, basophils, other Th2 cells or NK1.1$^+$ cells. The IL-4 is opposed by IFNγ. This opposition is blocked by antibodies to IFNγ. The sources of

IFNγ are T cells and NK cells. Priming for Th0 differentiation to Th1 is strongly enhanced by IL-12 about ten-fold (although IL-12 is not necessarily required), but inhibited by IL-4. Culture of Th0 clones with IL-12 and anti IL-4 resulted in only IFNγ producing cells (Openshaw 1995). The major source of IL-12 is macrophages although dendritic cells also produce it at low levels. Thus, Th2 cytokines prime for Th2 cells and inhibit priming for Th1 cells, whereas Th1 cytokines prime for Th1 cells (enhanced by IL-12). These results demonstrated that the same cells could be influenced in either direction by cytokines and that therefore the direction was not predetermined as the lineage specific theory would suggest.

Whether individual cells *in vivo* can be classified as Th1, Th2, or Th0 as opposed to responses or populations being characterized as Th1 or Th2 and even whether there are other phenotypes is also debateable. Cells derived from hyperimmune subjects or cultured with certain cytokines may conform to the Th1 / Th2 model, but clones stimulated with mitogens or alloantigens frequently co-express Th1 and Th2. Activation of T cell clones with polyclonal activator or antigen, with subsequent measurement of intracellular cytokine (IFNγ and IL-4) of single cells by flow cytometry revealed some interesting findings (Openshaw 1995). This is a valuable study because it investigates the cellular response as opposed to the population response and it does not rely on mRNA which is not necessarily indicative of protein. They found that 96% of established Th1 clones stimulated with PMA and ionomycin exclusively produced IFNγ and that <2% produced IL-4. With established Th2 clones 30% produced IL-4 (low percentage speculated to be due to transient expression) and < 2% produced IFNγ. These results would seem to

suggest that established Th1 and Th2 responses are composed of actual Th1 and Th2 cells. The only shortcoming of this study is that it was done with clones instead of fresh cells.

Whether Th1 or Th2 responses or populations are terminally differentiated or have the capacity to shift is also debateable. Swain et al established that Th1 or Th2 lines derived from TCR transgenic mice retained their phenotype after transfer to normal mice (Swain 1994). However, Th2 cells stimulated with IL-12 were shown to produce IFNγ while still producing their Th2 cytokines. Studies with human PBMC demonstrated that Th1 purified protein derivative-specific T cells shifted to Th0 or Th2 if cultured with IL-4 and allergen-specific Th2 cells shifted to Th0 or Th1 if cultured with IFNγ and anti IL-4 (Maggi 1992). An important aspect of this study was that fresh naturally occurring cells were used as opposed to transgenic T cells or clones. It may not be a question of whether T cells have the capacity to shift, but when in their lifespan or differentiation do they have the capacity to shift. Not only is this topic debateable, but it is considered very important, because shifting the Th1/Th2 spectrum is viewed as a promising potential treatment for many disorders.

Each cytokine is independently regulated, which makes the Th1 Th2 paradigm difficult to explain. A model proposed by Ross 1994 and Kelso 1995 suggests that cytokines are independently regulated, but that certain regulators increase the probability of expression of some, but not all, cytokines. This theory would accommodate T cells which may synthesize only some of the cytokines of one subset. However studies which have found such cells may have assays without the sensitivity to detect some of the

cytokines.

Cytokine Influences on Ig Class Switch

Some of the functions of cytokines include inducing B cells to proliferate, synthesize immunoglobulin and switch the isotype of immunoglobulin that they produce. Of particular interest in allergy are cytokines which cause isotype switch to IgE. The first cytokine identified with this ability was IL-4. Exogenous IL-4 added to *in vitro* cultures of purified mouse B cells containing LPS or activated T cells induced increased IgE and IgG1. With human PBMC or with isolated B cells cultured with anti CD40 monoclonal antibody or activated T cells, IL-4 was shown to induce IgE and IgG4. In both murine and human systems IFNγ inhibits IgE production. One study compared the ability of IL-4 to induce IgE and IgG4 production from atopic patient PBMC versus healthy donor PBMC (Punnonen 1993). They found no significant difference in the IL-4 induced IgE or IgG4 in the two groups, nor in the ability of IFNα or IFNγ to inhibit the activity of IL-4 in the two groups, suggesting that there is not an inherent difficulty or hyperreactivity in atopic B cell responsiveness to these cytokines or their capacity to switch isotypes.

A more recently discovered cytokine, IL-13, has been shown to have some functions in common with IL-4. One of those common functions is the ability to switch B cells to IgE production. In studies with mature human B cells IL-13 was shown to induce IgG4 and IgE, as well as CD23 expression (Punnonen 1993). However IL-13 was two to four fold less effective than IL-4 at inducing IgE synthesis. In studies with immature fetal human B cells IL-13 induced proliferation, class switch and IgM, IgG and IgE synthesis (Punnonen 1994). The ratio of isotypes produced was similar to that of IL-4. Like IL-4,

these functions of IL-13 were enhanced in the presence of 100 U/ml IL-6 and inhibited in the presence of 100 U/ml IL-12, IFNα, IFNγ or TGFβ. Antibodies to IL-4 had no effect on IL-13 induced Ig synthesis indicating that it acts independently of IL-4. In contrast to IL-4, IL-13 induced Ig levels five to fifteen fold lower than those induced by IL-4 at an equivalent concentration of 400 U/ml. IL-13 also differed from IL-4 in that it required IL-7 which is known to stimulate T cells and IL-13 did not induce increased expression of CD23, CD40 or HLA-DR. The significance of the difference between IL-4 and IL-13 to induce CD23 expression by immature human B cells is unknown. CD23 is the low affinity IgE receptor and may be involved in some form of negative feedback mechanism. Soluble CD23 upregulated IgE secretion (Pene 1989; Delespesse 1989) and monoclonal antibodies to CD23 inhibited the IgE response (Pene 1988; Chretien 1990). In summary, IL-13 induces the same switches as IL-4, but is less efficacious at doing so. The relative importance of these two cytokines *in vivo* would have to take into account the relative concentrations and stabilities of the two as well. In this regard, the very high levels of cytokine used to elicit the *in vitro* effects described above should not be overlooked.

Th2 and Allergy

It has been speculated that the allergic response is a Th2-like response, with one of the major factors being IL-4. IL-4 appears to have many roles in inflammatory responses such as allergy. In combination with IL-3 and IL-10, IL-4 stimulates mast cells and basophils. It inhibits the activation of the Th1 response and subsequent cytokine production, stimulates macrophages to secrete IL-1 and as discussed at length above induces the B cell switch to IgE production.

Evidence for the association between Th2 responses and allergy exists. Studies have been reported in which (i) spontaneous cytokine mRNA (Esnault 1996); (ii) cytokine production in response to polyclonal stimulation (Tang1995; Esnault 1996); and (iii) cytokine production in response to antigen stimulation (Gagnon 1993; Imada 1995); were measured in atopic and non-atopic subjects. More Th2-like responses were seen from the atopic group.

Recent evidence contradicts the presumed relationship between Th2-like responses to antigen, IgE specific for the antigen and clinical sensitivity to that allergen. In particular the relationship between IL-4 and IgE has been questioned (Marsh 1994). This group was interested in the genetic factors which in part determine total serum IgE concentration, and the role of IL-4 in regulating T cell differentiation to Th2 and immunoglobulin switching to IgE. They hypothesized that polymorphism in the gene for IL-4 could be linked to IgE concentration. To test their hypothesis they measured the log of total serum IgE concentration and the log of multi allergen specific IgE concentration of 170 subjects from 11 families each with at least one atopic child. Then they performed a linkage analysis of the serum concentrations to genetic markers within the IL-4 gene cluster including markers within the IL-4 gene. The IL-4 gene cluster includes IL-13, IL-5 and other cytokine genes as well. What they found was significant evidence for linkage between IL-4 R1 as well as several other markers IRF1, IL-9, D5S393, D5S399 and log of total IgE concentration. They did not find a linkage between these markers and log of multi allergen specific IgE. Next they removed all subjects with detectable multi allergen specific IgE and repeated the linkage analysis. This time the linkage was stronger. Then

they analyzed the atopic set and found no evidence of linkage. They concluded that in non-atopic subjects the IL-4 cluster was linked to total IgE, but not allergen specific IgE and that the IL-4 gene cluster was not linked to either allergen-specific or total IgE in atopics.

Other contradictory evidence, in this case looking at cytokine gene expression levels rather than genetic polymorphism, comes from a study by Van der Pouw Kraan (1994). Here, they investigated the level of IL-4 gene expression and attempted to correlate it to *in vitro* IgE synthesis. They were looking for a B or T cell functional defect in atopic individuals, and therefore isolated peripheral blood lymphocytes from normal and allergic individuals, stimulated them with anti CD2 + IL-2 (polyclonal activation) and measured the cytokine and total IgE production (*in vitro*). When they plotted IL-4 concentration versus IgE concentration they found a correlation in the normal subjects but not the atopic subjects. They concluded that IgE production in atopic patients is not related to IL-4 production. My concerns regarding this study are that polyclonal activation does not reflect the response to allergen and that total IgE is not as relevant to allergy as the allergen-specific IgE.

POLYCLONAL ACTIVATION

There are several advantages to using antigen, as opposed to polyclonal activators such as PHA, to stimulate the cells for measurement of cytokine. One is that only the cells specific for the antigen respond to antigen, approximately 0.01-1.0 % of the repertoire, whereas virtually all cells respond to polyclonal activators. Another is that the receptor

through which activation is signalled by antigen is the same as the one activated *in vivo* by allergen exposure, whereas different receptors are responsible for activation signalling by polyclonal activators. When different receptors are used for activation the result can be qualitatively and quantitatively different (Imada 1995).

The difference in results obtained by antigen stimulation and polyclonal activation was shown directly by Imada et al. in a study of grass pollen allergic and non-allergic subjects. With polyclonal activation the response elicited from the normal and atopic groups was the same for both populations. However, use of different polyclonal activators led to substantially different responses. PHA elicited a Th2-dominated response from both groups; while anti-CD3 elicited a Th1-dominated response from both groups. However, stimulation with grass pollen, the allergens to which these subjects were environmentally exposed, elicited a Th1 response from the non-allergic individuals and a Th2 response from allergic subjects. These results underline the importance of using antigen stimulation.

IMMUNE RESPONSES OF NON-ALLERGIC INDIVIDUALS

Do non-allergic individuals mount an immune response to allergens? This is a question which went unaddressed for a long time in the study of allergy. It was assumed that clinical non-responsiveness indicated immunological non-responsiveness. Due to this assumption the vast majority of earlier studies did not include non-atopic control subjects.

Proposed reasons for an immunological non-responsiveness include absence of appropriate immune response genes, lack of T cell or B cell receptors specific for the allergen or tolerance.

There is a hereditary factor to allergy which is unexplained, but does implicate genetics. Upon discovery of HLA genes, which largely determine responsiveness to a particular antigen, many efforts have focussed on associating HLA genes with a predisposition to allergy. In particular, associations of certain HLA-D allele frequencies with IgE responses to a particular allergen have been extensively sought. Some earlier studies found evidence for association of HLA-DR alleles with pollen allergens, but in contrast to a broad range of autoimmune disorders where linkage disequilibria have been readily demonstrated, association of clinical allergy with specific HLA alleles has been only poorly successful. A large, more recent study (Young 1994) of over 400 related atopic individuals and over 600 control subjects for allergy to *Dermatophagoides pteronyssinus* (Der p) 1 and 2, *Alternaria alternata* (Alt a) 1, *Canis familiaris* (Can f) 1, Fel d 1, and *Phleum pratense* (Phl p) 5 found only two weak associations between HLA-DR allele frequencies and IgE production to allergens. These associations were between Fel d 1 and HLA-DR-01 and between HLA-DR4 and Alt a 1. There were no associations found between HLA-DP allele frequencies and IgE responses. Many different earlier studies results were in conflict with each other in regard to conclusions about associations, likely due to small sample sizes and differences in population exposure. In fact, the only consistently observed association was that of *Ambrosia artemisifolia* (Amb a) 5 and HLA-DR2. Therefore the genes or alleles relevant to allergy are yet to be identified.

More recent studies have included non-atopic subjects as controls. The results have been mixed. Some studies found undetectable levels of allergen-specific antibodies of the IgE and IgG4 isotypes in non-allergic individuals (Peng 1990), but others found similar IgG1 levels in allergic and non-allergic subjects, as well as lower but detectable IgG4 in non-allergic subjects (Kemeny 1989).

Not only antibodies, but cytokine responses of non-allergic individuals have also been studied. Again, the results have been mixed. This is in part, likely due to different activation methods. Tang et al. studied the IL-4 and IFNγ responses to polyclonal stimulation with 10 μg/ml PHA. They found that non-allergic subjects produced significantly lower IL-4 and higher IFNγ than atopic asthmatic subjects, but not significantly different levels compared to non-atopic asthmatic subjects. Van der Pouw Kraan et al. also studied the response to polyclonal activation with anti CD2 and anti CD28 or anti CD2 and recombinant IL-2. They found no difference in the IL-4 or IFNγ produced by allergic or non-allergic individuals. These studies with polyclonal activation do not answer the question of what cytokine response, if any, is generated by antigen-specific immune cells of non-allergic individuals or even whether they exist. They only address the question of whether the whole repertoire of immune cells in allergic individuals are biased toward a Th2 response in comparison to non-allergic individuals.

In 1995 Imada et al. reported significantly different responses of grass pollen sensitive and non-sensitive individuals to grass pollen stimulation of fresh cells. This method of stimulation is much more appropriate for detecting differences in antigen-specific responses. Although 24 of 26 non-sensitive subjects produced undetectable IL-4,

19 of 26 produced detectable IFNγ. In fact, this IFNγ response was significantly higher than that of sensitive subjects. The sensitive subjects produced significantly higher IL-4, giving them a significantly lower IFNγ : IL-4 than the non-sensitive subjects. Gagnon et al. (1993) found that during the pollen season the Lol p 1 stimulated PBMC of atopics produced higher IL-4 and lower IFNγ than the PBMC of non-atopic subjects. However, out of season stimulation resulted in no IL-4 from either group and IFNγ from most subjects (all except for one atopic) which was not significantly different between atopics and nonatopics. Thus, in both allergen-specific activation studies most non-allergic individuals responded to the allergens with detectable cytokine production, but the response was more Th1-like than the allergen-induced cytokine responses of allergic individuals.

THE CONTROVERSY SURROUNDING THE ROLE OF IgG4 IN ALLERGY

IgG4 is a minor subclass of IgG, present at 0.4 mg/ml in human serum compared to 8 mg/ml IgG1. The half-life in plasma is 21-23 days. It has a short hinge region and does not fix complement well. IgG4 binds to FcγRI and FcγRII with lower affinity than either IgG1 or IgG3. It is said to have little clinical significance, however following repeated immunization the levels rise, and after chronic stimulation IgG4 is thought to be the dominant IgG subclass (Aalberse 1983).

The role of the IgG4 isotype antibody in the state of atopy is very controversial.

The theories range from it being an anaphylactic antibody supported by the evidence that IgG4 can elicit histamine release from basophils (Fagan 1982), to IgG4 being a protective blocking antibody which neutralizes the allergen before it binds to IgE bound on mast cells. The idea that IgG4 is protective has arisen from observations that specific IgG4 increases during allergen immunotherapy for bee venom (Muller 1989), dust mite (Tsai 1990), and ragweed (Peng 1990). It is interesting to note that the anti-PLA IgG response of novice asymptomatic beekeepers became IgG4 dominated over time as exposure increased (Aalberse 1983). Specific IgG4 is higher in allergic subjects to begin with. This has been shown in bee venom (Muller 1989), dust mite (Tsai 1990, Kemeny 1989, Jeanin 1994), wheat flour (Tikkainen 1989), grass pollen (Kemeny 1989, Jeanin 1994), ragweed (Peng 1990) and cat (Jeanin 1994) which must question its protective powers. Particularly since the value of immunotherapy itself is questionable, it is questionable to conclude that a factor increased by immunotherapy is protective. Notably, IgG4 increased in one immunotherapy study where clinical sensitivity decreased ie. good clinical results (Muller 1989) and in another study where the clinical results were poor (Tsai 1990).

The source of the increased IgG4 has been speculated about. It has been shown that IgG4 is enhanced by repeated exposure and that after chronic stimulation it is the dominant IgG subclass (Aalberse 1983). This would explain the increase in IgG4 during immunotherapy. It was suggested that IgG4 is indicative of exposure and that possibly increased IgG4 in allergic individuals indicated a more penetrable mucous membrane in this population (Kemeny 1989). It has also been suggested that the allergic population has abnormal isotype regulation (Jeanin 1994), yet only the IgG4 specific for the allergen was

elevated in this group compared to the non-allergic population. In contrast, the total IgG4 was not elevated in the allergic population.

An interesting study regarding the specificity of IgG4 and IgE antibodies was done by Kobayashi et al. They generated overlapping peptides of the Der p 2 mite antigen and tested the antibodies for binding to the different peptides. What they found was that IgG4 antibodies bound to a different subset of peptides than IgE antibodies isolated from the same sera of 13 patients. They thus concluded that the specificity of IgG4 and IgE antibodies are different.

Other studies have also investigated the specificity of IgE versus that of IgG4 (Schneider 1994). Human polyclonal IgG4 (of 14 bee keepers) and IgE (of 14 bee venom allergic patients) differed in their ability to bind phospholipase A_2 once it had been acetylated at the lysine residues. The IgG4 binding avidity dropped more substantially than the IgE binding avidity. The authors suggested that IgE is more heterogeneous in its epitope specificity, however the difference in the subjects was not considered.

Another interesting property of allergen specific IgG4 is whether or not it is associated or correlated to allergen specific IgE. An association was noted by Hammarström in 1987. An immunotherapy study reported in 1992 by Peng et al. found a correlation between ragweed specific IgE and IgG4 in atopic subjects only after 2 years of immunotherapy. The correlation was not present initially or even after one year of immunotherapy. While immunotherapy proceeded, the IgG4 to IgE ratio increased from 0.11 initial to 1.77 after one year to 41.0 after 2 years immunotherapy when the correlation appeared. Jeanin et al. report correlations of specific IgE and IgG4 to Der p,

grass pollen and cat dander in Der p allergic subjects. However, to achieve this correlation they had to exclude allergic subjects with low levels of IgG4. This casts doubt on the credibility of the correlation. Not only is it unclear whether specific IgE and specific IgG4 correlate in allergic individuals, but none of these studies report the status of the association in non-allergic subjects, making it difficult to put in context the importance of this possible correlation.

INTERLEUKIN 15

Discovery

Interleukin 15 (IL-15) was discovered by Grabstein *et al* in culture supernatants of a simian kidney epithelial cell line, CV-1/EBNA. The supernatants supported proliferation of the CTLL murine T cell line which is dependent on IL-2, thus characterizing the supernatant as containing a T cell stimulatory activity. IL-15 was isolated from the supernatants by hydrophobic affinity and anion exchange chromatographies, high pressure liquid chromatography and SDS- polyacrylamide gel electrophoresis, identifying it as a novel cytokine.

Structure

IL-15 is a 14-18 kDa cytokine with a four helix bundle structure and is therefore characterized as a member of the hematopoietin cytokine family (Grabstein 1994). Other members of this family are IL-2, 3, 4, 7, 9, 11, 13, GM-CSF, and EPO. The location of IL-15 was mapped to band 4q31 of chromosome 4 in the human genome (Anderson 1995). This is the same band which contains the IL-2 gene as well as other cytokines, chemokines and growth factors. Human IL-15 has 73 and 95 % identity with murine and simian IL-15 respectively (Grabstein 1994).

Function

Immediately since its discovery IL-15 has been compared with, and tested for similarities and differences to IL-2. These two cytokines do not share sequence homology, but the discovery of IL-15 itself revealed a common effector function, namely

T cell stimulation. At the same time it identified a different cellular source, epithelial cells. Several other functions of IL-15 have been discovered since that time. They include the abilities to activate natural killer (NK) cells (Carson 1994), costimulate human tonsillar B cells to proliferate and produce immunoglobulin (Armitage 1995), chemoattract T cells (Liew 1995), support the growth of antigen dependent T helper and cytotoxic clones, $\gamma\delta$ T cells (Nishimura 1996), tumor derived activated T cells (Lewko 1995), and activated normal T cells, and induce cytolytic effector cells. These functions are common to IL-2. However, the efficacy of the two cytokines, as well as their effect on other cytokine levels is not always identical. These details are discussed further in the section Effects of IL-15 Treatment *in vitro*: Comparison with IL-2. In summary, IL-15 is a hematopoietin which has many functions in common with IL-2, but no sequence homology. Perhaps the key difference between IL-2 and IL-15 is not functional but the cells which produce it; IL-2 being made by T cells (which are incapable of making IL-15) and IL-15 being made by fibroblasts and monocytes, which are incapable of making IL-2.

IL-15 Receptor

The IL-15 receptor (IL-15R) is a heterotrimeric complex composed of the IL-15Rα chain, the IL-2Rβ chain and the γ_c chain. The γ_c chain is also part of the IL-2, IL-4, IL-7 IL-9 and IL-13 receptors. The IL-2Rβ is only known to be a component of the IL-2 and IL-15 receptors. IL-15Rα is only known to function in the IL-15R. This component has high structural homology to the IL-2Rα component of the IL-2R. Both α

receptor chains have a signal peptide, Sushi domain(s), linker, proline-threonine-rich region, transmembrane region and a cytoplasmic tail. The IL-15Rα chain has a longer cytoplasmic tail than the IL-2Rα chain and contains only one Sushi domain compared to 2 Sushi domains in the IL-2Rα chain. Very striking and possibly very important is the fact that the affinity of IL-15 for IL-15Rα is one thousand-fold stronger than the affinity of IL-2 for the complete IL-2R.

Originally, the similarities in function and the demonstration that IL-15 inhibited binding of ^{125}I-IL-2 to the human NK-like cell line YT (Grabstein 1994), suggested common binding sites for IL-2 and IL-15. Antibodies 2A3 (anti human IL-2Rα) and Mikβ1 (anti human IL-2Rβ) were added to assays measuring ^{125}I-IL-15 binding and recombinant human IL-15 (rhIL-15) induced proliferation of PBMC, or generation of LAK and CTL activity. 2A3 had no effect, but Mikβ1 strongly inhibited IL-15. These results suggested that IL-2Rβ, but not IL-2Rα is part of the IL-15R. Subsequently, a murine pro-B cell line BAF/B03 expressing IL-2Rα and γ_c, but little or no IL-2Rβ chain were transfected with the human IL-2Rβ chain (Giri 1994). The resultant BAF/β cells proliferated in response to rhIL-15 and rhIL-2, confirming a role for IL-2Rβ in the IL-15R. The question remained whether IL-2Rβ is involved in binding IL-15, cell signalling or both. COS cells were transfected with IL-2Rβ alone and showed no binding of IL-15, nor did COS cells transfected with the γ_c alone.

After subsequent discovery of IL-15Rα, a more definitive experiment was conducted to elicit the true role of IL-2Rβ in the IL-15R (Giri 1995). A subclone, 32D-01, of the murine T cell IL-3 dependent cell line 32D which constitutively expresses the

IL-2Rα and γ_c chains, was selected for its loss of IL2Rβ chain expression. This subclone bound IL-2 but not IL-15. When transfected with IL-2Rβ, it bound IL-2 and proliferated in response to IL-2, but did not bind or proliferate in response to IL-15. 32D-01 transfected with IL-15Rα bound both IL-2 and IL-15, but did not proliferate in response to either. When transfected with both IL-2Rβ and IL-15Rα it bound IL-2 and IL-15 and proliferated in response to both. Thus in summary, these experiments defined the role of IL-2Rβ as a signalling, and not a binding chain. In fact, IL-15Rα alone is enough to bind IL-15 with high affinity. BAF cells were found to express IL-15Rα mRNA, which explains the ability of the BAF/β cells to bind IL-15. The apparent contradiction of the ability of the Mikβ1 antibody to abolish IL-15 binding may have been due to steric hindrance.

It was reasonable to suspect that the γ_c was also part of the IL-15 signalling process. The IL-2Rβ and γ_c are associated with the tyrosine kinases Jak1 and Jak3. The experiment with the 32D-01subclone demonstrated that the γ_c is not sufficient for signalling without the IL-2Rβ chain, but it was also shown that the IL-2Rβ chain was not sufficient for signalling without the γ_c in an experiment using L929 murine fibroblast cells (Giri 1994). These cells were transfected with IL-2Rα and IL-2Rβ yet showed no increase in c-fos or c-myc proto-oncogene expression when exposed to IL-15. In contrast when the same cells were transfected with IL-2Rα, IL-2Rβ and γ_c the IL-15 treatment produced an increase in the proto-oncogene expression levels. It has been suggested that binding of IL-15, like that of IL-2, dimerizes the two chains which results in activation.
Another role for the γ_c was suggested by work done with the X linked Severe combined

immunodeficiency (X-SCID) mouse model (Kumaki 1995). COS-7 cells which were transfected with the IL-2Rβ chain alone or with the IL-2Rβ and mutant γ_c from X-SCID patient did not internalize IL-15. However, COS-7 cells transfected with the IL-2Rβ and intact γ_c did internalize IL-15, suggesting that the γ_c is responsible. In conclusion, it is the IL-15Rα component which binds IL-15, the IL-2Rβ and γ_c which are involved in cell signalling and the γ_c which is crucial for internalization of the cytokine.

EXPRESSION OF IL-15 AND IL-15R

IL-15 mRNA has been detected in placenta and skeletal muscle and at lower levels in kidney, lung, liver, heart and pancreas (Grabstein 1994). The best cellular source of IL-15 is adherent peripheral blood mononuclear cells, however the epithelial cell line CV-1/EBNA and the fibroblast cell line IMTLH also produce detectable IL-15 mRNA. In contrast, the main cellular source of IL-2 is activated peripheral T cells.

The mRNA expression of the IL-15 Rα component detected in the liver was very high and moderate levels were detected in spleen, lung, heart, kidney, and skeletal muscle. The highest expression of IL-2 Rα mRNA was seen in the spleen, with high levels also seen in the skeletal muscle, lung and heart. Only moderate levels were detected in the kidney and testis and very low or undetectable levels in the liver. The T cell lines CTLL and D10 produced high levels of IL-15 Rα mRNA. Bone marrow and thymic stromal lines, a macrophage line and two B cell lines also produced mRNA of IL-15 Rα. IL-2 Rα mRNA expression was also high in the T cell lines, and present in the macrophage and B

cell lines, but not present at all in the stromal lines. Like IL-2 Rα expression, IL-15 Rα expression increases upon cell activation (Giri 1995).

The differences in the expression patterns of IL-2 and IL-15, and between their respective receptor components IL-2 Rα and IL-15 Rα suggest that IL-2 and IL-15 may play different functional roles *in vivo* despite showing such similar effector functions *in vitro* and using common receptor subunits. The biological relevance of the different expression patterns can only be speculated about at this time. Given the different cellular sources of the two cytokines it appears plausible that they are subjected to different regulatory factors. Giri *et al* suggest that IL-2 and IL-15 may act on different populations of lymphocytes or at different stages of lymphocyte differentiation.

Production and Regulation

I have already discussed which cell types and tissue types produce IL-15 mRNA, however the level of IL-15 synthesis and the conditions or factors which stimulate and regulate its synthesis are less well characterized. Doherty et al. have found constitutive IL-15 mRNA in mouse peritoneal and bone marrow derived macrophages. They also found that IL-15 mRNA and protein levels were higher after stimulation with Bacillus Calmette Guérin (BCG) or LPS and optimal if cells were treated with IFNγ prior to stimulation with either microbial agent. The effect of macrophage inhibitory cytokines IL-4, IL-10, IL-13 and TGFβ on IL-15 production was also tested. In the presence or absence of BCG these cytokines did not inhibit IL-15 mRNA although they did inhibit production of IL-12 (another macrophage produced cytokine). The only inhibitory effect on IL-15 production seen occurred when macrophages were pre-treated with any of the

inhibitory cytokines in addition to IFNγ followed by BCG stimulation. The result was partial inhibition of IL-15 to the levels seen in response to BCG stimulation alone, suggesting that the cytokines had an antagonistic effect on IFNγ priming.

All the IL-15 expression thus far presented has been IL-15 mRNA, however, mRNA is not necessarily translated into protein. Indeed there is substantial evidence that IL-15 mRNA is frequently produced at high levels without translation or secretion of biologically active protein (reviewed in Tagaya 1996). Therefore which cells produce and release functional IL-15 protein and which stimuli promotes its release remains unknown. There has been one report by McInnes et al which investigated IL-15 protein levels in synovial fluids of arthritis patients. IL-15 was measured in 17 rheumatoid arthritis patients and 6 osteoarthritis patients. All OA patient synovial fluid contained <10 ng/ml IL-15, but IL-15 levels in RA patients ranged from 0-1200 ng/ml. The significance of this data cannot be clear unless the IL-15 levels of non-affected individuals is known, yet at this time the IL-15 protein levels in any other cell population of any species is unreported in the literature. This is a critical gap in the study of this cytokine.

In our studies, described below, we examined the *in vitro* levels of IL-15 protein produced by PBMC of allergic and non-allergic individuals stimulated with (i) allergen or (ii) polyclonal activators or (iii) nonstimulated.

EFFECTS OF IL-15 TREATMENT *IN VITRO* : COMPARISON WITH IL-2

While the proliferative response of murine γδ T cells to optimal doses of IL-15 or IL-2 is similar, the minimum and optimal doses differ (Nishimura 1996). Less than 1 ng/ml IL-2 causes proliferation and 100 ng/ml is optimal, yet at least 10 ng/ml IL-15 is required and 1000 ng/ml is optimal. The cytokine production also differs (Nishimura 1996). Less IFNγ, IL-4 and TNFα are produced in response to IL-15 (1000 ng/ml) than IL-2 (1000 ng/ml), and no IL-10 is produced in response to recombinant IL-15 (rIL-15) in contrast to IL-2 administration.

Cytokine production by human tumor infiltrating lymphocytes (TIL) also differed depending on whether IL-15 or IL-2 was used to support their proliferation (Lewko 1995). IL-2 administered at 60 or 6000 U/ml stimulated IFNα, GM-CSF and TNF. IL-15 administered at 100 ng/ml did not effect TNF but stimulated more IFNα and GM-CSF than IL-2 alone or even IL-2 in conjunction with IL-15. The proliferative response and tumor cell loss were similar in IL-2 treated and IL-15 treated cultures.

The optimal dose of IL-15 or IL-2 required for human NK cell proliferation is the same (10 ng/ml), but the minimum dose for IL-2 is 0.1 ng/ml whereas for IL-15 the minimal dose is 1 ng/ml (Carson 1994). Induced cytotoxicity and antibody dependent cellular cytotoxicity are very similar for both cytokines, as is production of IFNγ and TNFα. However, GM-CSF levels stimulated by IL-15 are approximately half the levels stimulated by IL-2.

With an *in vivo* murine model McInnes et al have shown that one injection of 500 ng rIL-15 induces a local inflammatory infiltrate which is predominantly composed of T cells (up to 56%). They also showed with phase contrast microscopy that rIL-15 (10 - 1000 ng/ml) induced *in vitro* polarization of human T cells purified from blood. Anti IL-15, like anti IL-8 partially inhibited synovial fluid induced T cell polarization *in vitro* and collagen gel migration. When used in combination these antibodies completely inhibited polarization and migration suggesting that both IL-15 and IL-8 are important mediators of chemotaxis by synovial fluid.

PBMC of HIV-infected and non-infected individuals demonstrated enhanced proliferation in response to polyclonal (PHA), Ag-specific (HIV peptide), and recall Ag (tetanus toxoid) stimuli when treated with IL-15 at high concentration (1, 5 and 10 ng/ml) (Seder 1995). The HIV peptide and tetanus toxoid stimuli are particularly noteworthy because they elicit a more physiologic response than polyclonal stimulation. IL-2 at 1 ng/ml also enhanced proliferation in response to stimulation, and in some cases to a greater degree than IL-15. These doses of IL-15 did not affect IL-2, IL-4, or IFNγ protein production, nor did treatment with 1 ng/ml IL-2. At larger doses of IL-15 such as 100 and 300 ng/ml they report increased IFNγ production. An interesting addition to these studies would have been to study the effect of anti IL-15 treatment on PBMC proliferation. This approach would indicate whether endogenous IL-15 has an impact.

In our studies, we measured the IFNγ, IL-4, IL-5 and IL-10 responses of PBMC from allergic and non-allergic individuals stimulated by allergen, or nonstimulated, in the presence of IL-15 and IL-2.

THERAPEUTIC POTENTIAL OF IL-15

IL-15, like IL-2, may have therapeutic potential for conditions requiring a stronger cell-mediated immune defense. These are wide ranging potential applications indeed eg. cancer, AIDS, leprosy, persistent viral and intracellular parasitic or bacterial infections. IL-15 *in vitro* has activated cytolytic effector function of NK and T cells and therefore may stimulate innate and adaptive immunity. It has also been shown to increase Ig secretion and therefore may be important in humoral immunity.

Use of IL-2 is limited because of its toxicity. Any treatment which increases TNF production has its dangers, because too much systemic TNF can be fatal. However, local TNF is very desirable in the fight with tumors. IL-15 has induced less TNF from γδ T cells as well as TIL. IL-15 may be an attractive, less toxic alternative to IL-2, but are IL-15 and IL-2 so similar that they are interchangeable? I do not think so.

On the one hand it would not be surprising to learn that functions as crucial as those of IL-2 in stimulating and maintaining the immune system were duplicated in a second cytokine. Evolution could have selected this protective redundancy and several precedents are known for this for other cytokine combinations. However, given the different expression of IL-15 and IL-2, as well as IL-2Rα and IL-15Rα, and the new IL-15R reported on mast cells and monocytes (Tagaya 1996) I find this an unlikely explanation. All that is known about IL-15 function has been derived from *in vitro* studies and the *in vivo* role of IL-15 is only speculation. The downstream or environmental effects have not been addressed.

IL-15 has very high affinity for its receptor, is not inhibited by macrophage

inhibitory cytokines and appears to be very tightly regulated *in vivo* because it is virtually undetectable. Intuitively it appears IL-15 may be a particularly potent cytokine. In fact, the only report of IL-15 protein has been in rheumatoid arthritic patient synovial fluids, an undesirable condition. IL-15 is a long way from application in therapy.

Perhaps, at least in the near future, IL-15 may be most useful in stimulating cells *in vitro*. In this arena, the question of whether there are advantages of IL-15 versus IL-2 would lie in practicalities like availability, which is more potent or long-lasting, or easier or cheaper to produce.

SUMMARY OF THE PROJECT

In one project we have examined the relationship between (i) intensity of skin test reactivity, (ii) antibody responses, and (iii) allergen-specific cytokine production of allergic and clinically non-sensitive individuals to cat antigen. The intensity of the epicutaneous wheal, the antigen-specific IgE and IgG4 as well as total IgE, and the IFNγ, IL-4 and IL-10 production in response to cat antigen stimulation were compared between the two subject groups. The parameters were tested for correlations in the population as a whole and in selected subpopulations as defined by clinical sensitivity. The strength of this study rests on the sensitivity of the cytokine and IgE assays which allow us to measure the cytokine production by fresh human cells stimulated with antigen, as opposed to polyclonal stimulation methods or use of clones, and the examination of antigen-specific antibodies as opposed to total isotype production.

In a separate study we have examined the effects of exogenous IL-15 (10-1000 pg/ml) on cytokine production by human PBMC in recall responses. The stimulating antigen chosen was cat antigen at 500 BAU/ml, and the effects of exogenous IL-15 on cytokine production by unstimulated human PBMC were also studied to reveal any non-specific stimulation. The cytokines measured were IFNγ (representative of Th1), IL-5 (representative of Th2) and IL-10. The abilities of IL-15 to act independently of endogenous IL-2, and to synergize with exogenous IL-2 in affecting the cytokine production were also studied. In addition, IL-15 secreted by human PBMC spontaneously or in response to recall antigen (cat) or polyclonal activators (LPS, PHA) was sought, but not detected.

METHODS

Subjects

This study, approved by the University of Manitoba Faculty Committee on the Use of Human Subjects in Research, included 40 cat allergic subjects and 43 clinically non-sensitive subjects. The entrance criteria met by the cat allergic subjects included a history of cat-induced allergy symptoms (rhinitis, urticaria, angioedema or asthma), a positive epicutaneous test to a standardized cat hair extract (Allergologisk Laboratorium) containing Fel d 1, serum IgE indicated by a positive RAST of greater than 0.35 units and no history of immunotherapy. In contrast, the non-allergic subjects had no clinical history of cat allergy and negative results to the epicutaneous test and RAST. All subjects were between 14 and 50 years of age, gave written informed consent to participate and none were taking oral medications.

Blood Processing

Whole blood (25 ml) was collected into 1 ml of 2.7% ethylene diamine tetraacetic acid (EDTA). Blood was diluted to 40 ml with 0.15 N NaCl and centrifuged with Histopaque-1077 (SIGMA) for 30 minutes at 1600 rpm. Plasma samples were collected from the top layer and frozen at -20^{0}C. Immediately following plasma collection, peripheral blood mononuclear cells (PBMC) were collected from the interface, washed three times with 0.15 N NaCl, counted with Trypan Blue exclusion and frozen under liquid N_2. The freezing solution contained 50% fetal calf serum (FCS), 42.5 % RPMI 1640 and 7.5 % dimethyl sulfoxide and was cooled on ice before use.

Non-idet P-40 (Sigma) is a non-ionic detergent shown to inactivate HIV and other

envelope viruses. For safety reasons the possibility of treating the plasma samples with NP-40 was tested. Aliquots of six allergic subject plasma samples were treated with 0.5% or 2.5% NP-40 and compared to untreated aliquots in terms of response in the ELISAs. The lack of an impact from NP-40 addition resulted in subsequent treatment of all plasma samples with 0.5% NP-40.

ELISA Reagents/Buffers

The plates used for all enzyme linked immunosorbent assays (ELISA) were 96 well flat bottom (Corning). All capture reagents were diluted in bicarbonate buffer of pH 9.6. After coating, plates were blocked with a phosphate buffered saline (PBS) buffer containing 1% bovine serum albumin (BSA), and NaN_3 0.02%. All samples, standards and developing reagents were diluted in PBS buffer containing 0.5% BSA, 0.05% Tween 20, and NaN_3 0.02% with a final pH of 7.4. The substrate p-nitrophenyl phosphate was dissolved in buffer containing $MgCl_2$-$6H_2O$ dissolved in diethanolamine at pH 9.8. The ELISAs for IgE used the same coating buffer, but were blocked with PBS buffer containing 2% BSA and NaN_3 0.02% and samples, standards and developing reagents were diluted with PBS buffer containing 0.2 % BSA, 0.05% Tween 20 and NaN_3 0.02%.

Most of the ELISAs required streptavidin alkaline phosphatase. It was always used at 0.0625 ug/ml as this concentration was found to be optimal in preliminary experiments.

All ELISA results were obtained by reading the absorbance at 405 nm and subtracting background absorbance at 690 nm.

Antibody ELISAs

Cat-specific IgG4 levels in the plasma were determined by ELISA using 50 µl of 50 µg/ml of a Fel d 1 containing cat hair extract to coat the plates. The standard was a particular non-allergic subject plasma sample arbitrarily assigned a value of 1000 U/ml. The detection reagents were a 5 µg/ml monoclonal biotin conjugated mouse anti human IgG4 (PharMingen) in combination with streptavidin-alkaline phosphatase (Jackson Immunoresearch).

Cat-specific IgE was similarly determined by ELISA capturing with 2 µg/ml of the standardized cat hair extract (ALK). The standard was a serum sample assigned a value of 1000 U/ml for comparison purposes. The developing antibodies were a goat IgG (anti human IgE; affinity purified myeloma originally provided by Dr. N.F.A. Adkinson Jr.) and a rabbit (anti goat IgG) alkaline phosphatase conjugate (Jackson Immunoresearch).

Total plasma IgE levels were also determined. For this assay the capture reagent was a monoclonal antibody against human IgE (clone 7.12, originally provided by Dr. A. Saxon) and the developing agents were the same as those described in the cat-specific IgE ELISA. The standard in the assay is human IgE (Pharmacia) serially diluted from 10 U/ml.

The reagents for the cat-specific IgE and total IgE ELISAs were provided by Dr. Zhikang Peng. We rigorously tested the isotype specificity of the developing antibodies

and the monoclonal coating antibody by coating plates with 2 μg/ml of IgG, IgM, IgE, IgA and ovalbumin each separately and developing with a range of developing antibody concentrations. The concentrations with minimal response to inappropriate isotypes and maximal response to IgE were selected for further usage. The monoclonal antibody was coated at a range of concentrations, followed by 2 μg/ml samples of either IgG, IgM or IgE and developed with the previously optimized concentrations of developing antibodies. This confirmed the specificity of the monoclonal coating antibody. The optimal concentration of all the other reagents used in all the assays described above was also determined in optimization assays carried out at the initiation of the project.

Cell Culture

Peripheral blood mononuclear cells (PBMC) were cultured in round bottom 96 well plates at 3.0×10^5 cells per well in a final volume of 200 μl per well incubated at 37°C and 5% CO_2 loosely wrapped in foil. The culture media was 10% fetal calf serum(FCS) in RPMI 1640 supplemented with 10 mM L-glutamine and 2×10^{-5} M 2-mercaptoethanol. Antigen-stimulated cultures contained the standardized cat hair extract (ALK) at a concentration of 500 Biological Allergy Units per ml (BAU/ml) which was equivalent to 26.5 μg/ml total protein. Cultures supplemented with recombinant IL-2 contained 5 or 10 WHO IU/ml IL-2 (Cetus). The optimal cell and antigen concentrations were determined by Dr. Mie Imada of this laboratory before my involvement in the project, and later confirmed by myself.

Culture supernatants were harvested and frozen at -20°C at 48 hours for IL-10

analysis, at 72 hours for IL-4 analysis and 96 hours for IFNγ analysis. These times were found to produce peak production of the respective cytokine in initial assays with multiple harvest times employed.

Cytokine Assays

Initially, IFNγ in the bulk culture supernatants was detected by a sandwich ELISA using purified polyclonal rabbit anti human IFNγ for capture and a monoclonal antibody cocktail of MIF 3009 and MIF 3125 purified and biotinylated for developing followed by streptavidin alkaline phosphatase. Later, PharMingen monoclonal antibodies were employed instead. The coating antibody was 1.5 μg/ml purified mouse anti human IFNγ and the developing antibody was biotinylated mouse anti human IFNγ used at 0.5 μg/ml, concentrations we found to give maximal sensitivity. The standard against which all samples were calculated was culture supernatant of PBMC stimulated with IL-12, Ag, and IL-2 , standardized against recombinant IFNγ standard (WHO). It was serially diluted from 40 U/ml. The detection limit was routinely 0.6 U/ml or less.

IL-10 was also detected by a sandwich ELISA. Monoclonal rat anti human IL-10 served as the capture reagent at 2 μg/ml and a biotinylated monoclonal rat anti human IL-10 against a different epitope served as the developing reagent, followed by streptavidin alkaline phosphatase. The standard was recombinant IL-10 diluted in culture media. It was serially diluted from 1000 pg/ml. The detection limit was routinely 16 pg/ml or less.

IL-5 was measured by a sandwich ELISA. The capture reagent was 2 μg/ml purified rat anti human / mouse IL-5 monoclonal antibody (PharMingen). The developing

antibody was a biotinylated rat anti human IL-5 monoclonal antibody used at 1 μg/ml. The standard in this assay was recombinant human IL-5 (PharMingen) used at 1000 pg/ml. The detection limit of the assay was routinely 8 pg/ml.

IL-4 was measured by a bioassay using the cell line CT.h4S (initially provided by Dr. W. Paul NIH / NAIAD). This cell line was maintained in a culture media containing 10% FCS in RPMI supplemented with 8 ng/ml IL-4. Within 16-24 hours prior to the assay, the cells were split to ensure that they were in log phase growth at the time of application. The cells were washed immediately before application in the assay and resuspended in culture media at 0.12×10^6 cells/ml. They were added to an equal volume of culture media in 96 well flat bottom plates. Culture supernatants were applied to sample wells at a final dilution of 1/3 and serially diluted twofold. Standard recombinant IL-4 was added to standard wells at a final concentration of 40 pg/ml and serially diluted twofold to 0.3 pg/ml. Control wells for each sample had anti IL-2 added to ensure that the cell response was not due to IL-2 instead of IL-4. Selected assays were carried out plus and minus neutralizing anti IL-4 polyclonal antibody (Sterling Prognostics). The assay was incubated 40 hours at 37°C before adding the (3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) solution. The MTS solution consisted of 2/3 RPMI 5% and supplemented with PSF (penicillin G sodium, streptomycin sulfate, amphotericin B), 1/3 MTS 1 mg/ml and 0.008% Phenazine Methosulfate (PMS) 0.92 mg/ml. The assay was incubated 24 hours longer at 37°C wrapped in foil because MTS is light sensitive. After the incubation the absorbance was measured at 492 nm subtracting background absorbance at 690 nm. Routinely the assay sensitivity was between 1 - 2.5

pg/ml.

IL-15 was detected by the same bioassay procedure using the CTLL except the first incubation is only 18 hours. The assay was carried out in the presence of 1/7000 anti IL-2 (Cetus) to establish that the response was not due to IL-2. Under these conditions the sensitivity to IL-2 is 5 U/ml and the sensitivity to IL-15 is 10 pg/ml.

Monoclonal Antibody Preparation

Hybridoma cell lines were purchased from American Type Culture Collection, thawed in a 37°C water bath and washed in thawing solution containing 20% newborn calf serum. Then they were resuspended in the recommended media, grown to sufficient volume and allowed to exhaust (<50% viable, typically 20-30%). The cells were removed from the exhausted suspension by harvesting only the supernatant (containing the antibodies) after centrifugation.

The concentration of the monoclonal antibodies was determined by ELISA. This ELISA was a sandwich ELISA where the capture antibody was a sheep anti mouse IgG and the developing antibody was biotin-conjugated goat anti mouse IgG + IgM used at 5 μg/ml and 1/10000 respectively. The standard was mouse IgG.

After concentration determination, some of the monoclonal antibody preparations required concentrating by the Centriprep technique. A Centriprep 30 (Amicon) with a molecular weight cutoff of 30,000 Da was used. Some monoclonal antibodies were dialysed against serum free RPMI to remove metabolites and adjust pH.

Hybridoma Specificity and Culture Conditions

Hybridoma HB 180 was grown to exhaustion in 10% Fetal Calf Serum (FCS) / RPMI and produced murine IgG2a antibodies against HLA-DR, DQ ie. anti human MHC Class II. The hybridoma HB 96 was grown to exhaustion in 10% FCS / Dulbecco's minimum essential medium (DMEM) and produced murine IgG1 antibodies against HLA-D. The HB 116 hybridoma produced IgG antibodies against HLA-A, B, C (human MHC Class I) after being grown to exhaustion in 12% FCS / DMEM + HAT. The HAT supplement was 100 μM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine final concentrations in tissue culture. HB 6025 was exhausted in 10% FCS / RPMI media and produced IgG antibodies against human IgG4.

Blocking of Antigen Presentation in Culture

Attempts to block antigen presentation were conducted through cell culture techniques as previously described with the modification that monoclonal antibodies against the major histocompatibility complex (MHC) were added to the culture. A cocktail of antibodies against human MHC Class II produced by HB 180 and HB 96 was added at a final concentration of 18 ug/ml. An anti human MHC Class I monoclonal antibody produced by HB 116 was added to independent cultures at a final concentration of 50 ug/ml as a control. Other control antibodies include J11D, a rat IgM monoclonal antibody specific for mouse B cells, immature T cells, erythrocytes and neutrophils, the ligand for which is not expressed in humans; (ii) a mouse IgG1 monoclonal antibody against OVA; (iii) PK136, a mouse IgG2a antibody against mouse NK1.1, also not

expressed in humans; and (iv) YTS169, a rat IgG2b antibody against mouse CD8 that does not react with human CD8. The second method employed to block antigen presentation was to add chloroquine to cultures at final concentrations of 20 and 100 μM following methods previously described in detail (Imada 1995).

Production of IL-15 in Response to Antigenic and Polyclonal Stimulation

PBMC of two subjects, one cat allergic and one non-cat allergic were cultured in round bottom plates at 500,000 cells per well and final volume 200 μl stimulated with 0, 50 and 500 BAU of cat hair extract. PBMC of the same subjects were also cultured 200,000 cells per well, final volume 200 μl with the polyclonal activator lipopolysaccharide (LPS) at concentrations of 0.1, 1.0, and 10.0 ug/ml. Cells were kept on ice during counting prior to plating with the intention of minimizing adherence of the monocyte population to the plastic tube. Cultures were incubated at 37°C 5% CO_2. After the first hour of culture the plate was wrapped in foil to reduce evaporation. Supernatants of 100 μl were harvested at 36, 60, 84, and 108 hours from cat antigen stimulated cultures and at 6, 12, 24, 48 hours from LPS stimulated cultures, then applied to the CTLL bioassay.

PBMC from two different cat allergic subjects were cultured at 300,000 cells / well with and without stimulation by PHA (0.5%) and LPS (10 ng/ml) independently. Each stimulus was also cultured in the presence of IFNγ at three different concentrations (10, 1, 0.1 μg/ml) and independently in the presence of IL-4 at three different concentrations (1000, 100, 10 pg/ml). Cultures were incubated at 37°C 5% CO_2 for 10, 24, and 48

hours with supernatants being removed at those times. Supernatants were tested for the presence of IL-15 in the CTLL bioassay.

Cytokine Production by PBMC Cultured in the Presence of IL-15

PBMC from four cat allergic subjects were thawed and cultured at 300,000 cells / well, final volume of 200 µl with and without cat antigen stimulation at 500 BAU/ml, with recombinant human IL-15 (PeproTech) at 0, 10, 100, and 1000 pg/ml final concentration. The PBMC were also cultured with an anti human IL-2 antibody cocktail at **conc** , cat hair antigen (500 BAU/ml), and the same range of concentrations of recombinant IL-15. Cultures were incubated at 37°C 5% CO_2 and supernatants were harvested at 48 and 96 hours for IL-10 and IFNγ assays respectively.

PBMC from 2 nonallergic subjects were likewise thawed and cultured at 300,000 cells / well in a final volume of 200 µl stimulated with 0, 500 BAU/ml cat hair antigen and treated with 0, 100, and 1000 pg/ml final concentration. Some cultures were additionally stimulated with recombinant IL-2 of 5 U/ml (Cetus) and other cultures were treated with an anti IL-2 1/2500. Incubation and harvest procedures were the same as for the allergic subjects. Cultures of fresh PBMC were also done and the concentration of IL-2 was adjusted to 10 U/ml.

Statistics

All correlation statistics reported are two-tailed Spearman coefficients and significance values. To test for significant difference in any parameter between different

subject groups the Mann-Whitney U Test was used.

RESULTS

Immune Responses of Allergic and Non-Allergic Subjects to Cat Allergen

We are interested in characterizing the immune responses of allergic and non-allergic individuals to cat allergen in terms of Th1 and Th2 associated cytokines and plasma antibody isotypes. It is a widely held belief that allergy is associated with a Th2 type of response resulting from IL-4 induced switching of B cells to IgE production and that subsequent exposure to allergen leads to allergen binding to mast cell-bound-IgE and causes the degranulation of mast cells and release of mediators which cause the clinical symptoms of allergy. In this phase of the study we attempted to determine the strength of the association between these immunologic and clinical parameters.

For a means of examining the relationship between *in vivo* sensitivity (as approximated by the intensity of allergen-stimulated skin test reactivity) and patterns of cytokine synthesis elicited by the allergen, we stimulated patient-derived PBMC with cat allergen and measured antigen-driven IFNγ (one of a group of cytokines produced in a Th1-like response), IL-4 (one of a group of cytokines produced in a Th2-like response), and IL-10 (not characterized as either Th1 or Th2 in human clones). We also measured the IgE (total and specific for cat antigen) and IgG4 (cat-specific) antibody levels directly *ex vivo* in plasma. We then attempted to correlate the allergen-specific cytokine production, antibody production and clinical sensitivity of the subjects to cat allergen.

Skin Tests

The allergic subjects recruited for this study have a clinical history of cat allergy and tested positive to skin prick, intradermal skin tests and RAST tests with standardized

cat hair extract. In contrast, the non-allergic subjects have no clinical history of cat allergy and tested negative to the skin prick, intradermal and RAST tests to this allergen.

The intensity of the allergic wheal reaction was heterogeneous as influenced by the degree of clinical sensitivity. It was quantitated as the sum of the longest and orthogonal diameters of the wheal. This value was at least 6 mm greater than the diluent control. The wheal intensity data was provided by Dr. K. Simons (Pharmacology, University of Manitoba) and is shown in Fig 1.

Plasma Antibody

Plasma IgE and IgG4 levels were measured directly *ex vivo* by ELISA as described in Materials and Methods. These data are displayed in Fig. 2, 3 and 4. The units of cat-specific IgE and cat-specific IgG4 are from an arbitrary scale while total IgE is expressed as ng/ml. The bars depicted in the graphs represent medians, rather than means, because distribution medians are less influenced by outliers than means. There is considerable overlapping between the groups, which is a common phenomenon in human studies, because the subjects are from an outbred population. The difference between the two groups is significant for each isotype as determined by a Mann-Whitney U test. This non-parametric test was chosen instead of a parametric test (such as a t-test or ANOVA) because the values do not form a normal distribution according to a Kurtosis and Skewness test as well as the deviation of the allergic group and the deviation of the normal group were not always suitably equivalent. Both total IgE and cat-specific IgE are higher in the allergic population. Specific IgG4 appears to be significantly higher in the normal population, but it should be noted that this difference is on the borderline of significance.

Wheal intensity in response to epicutaneous skin test with cat antigen.

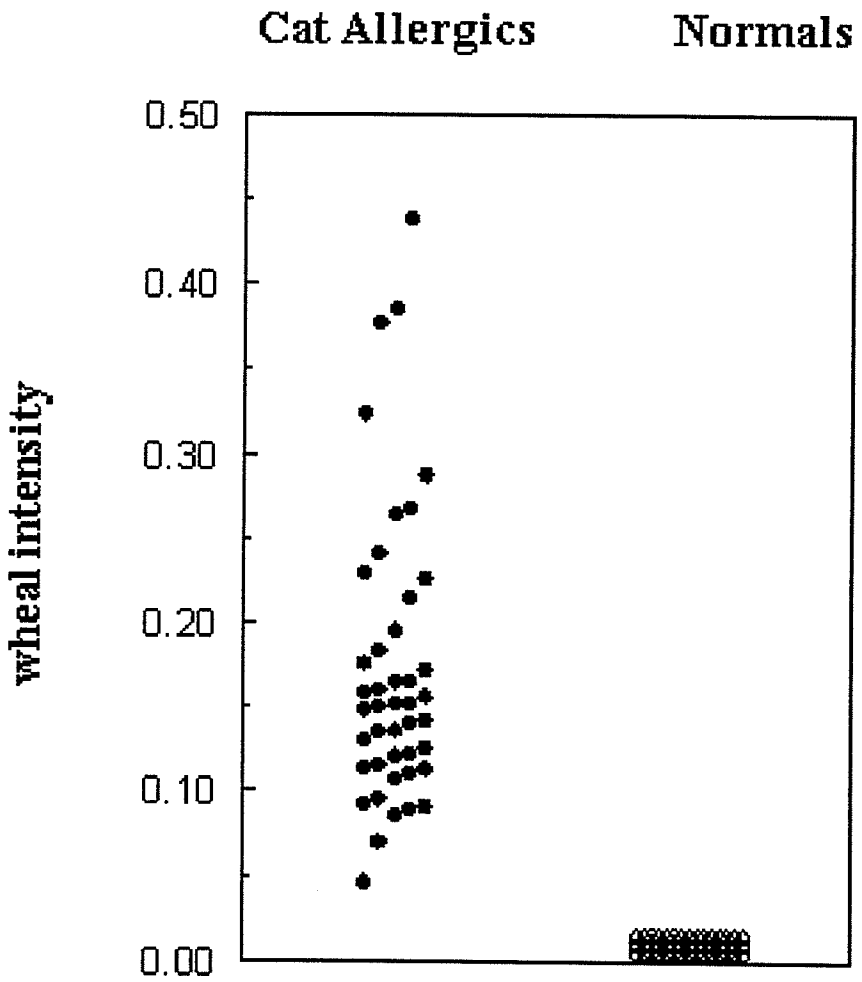

Figure 1. Cat antigen-induced wheal intensity.

Total IgE in Allergic and Non-Allergic Subjects

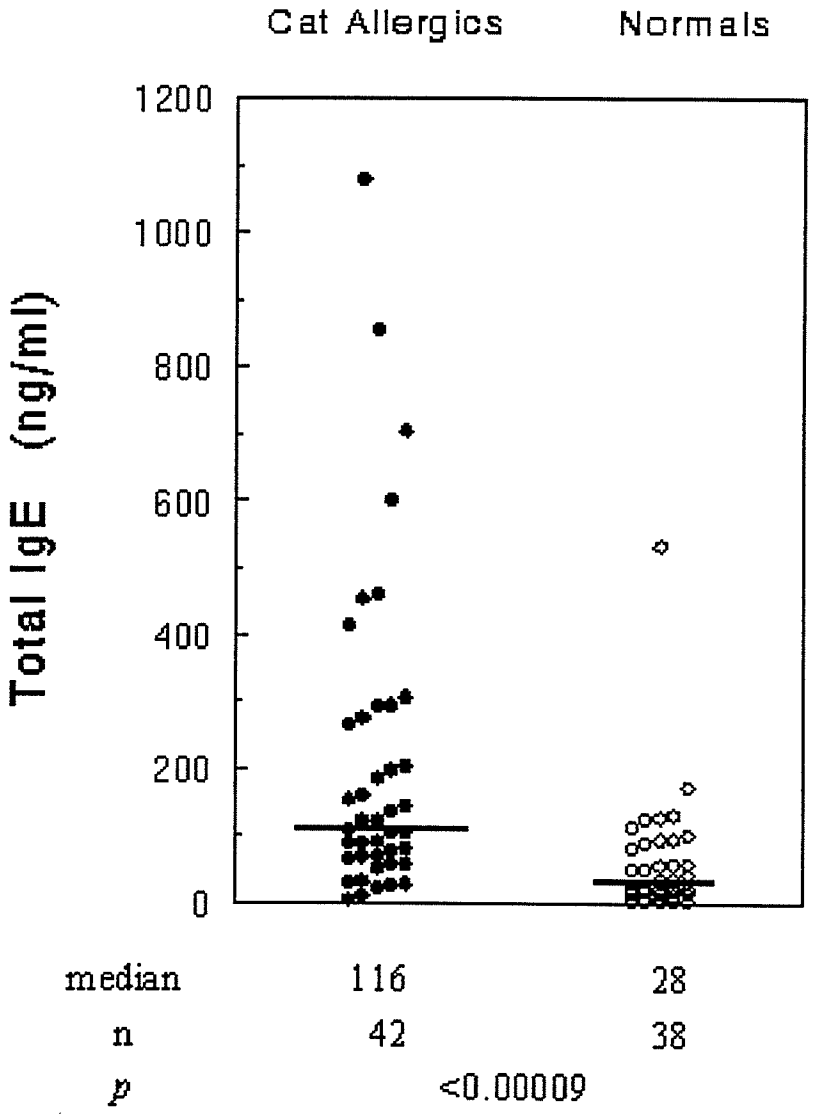

Figure 2. Total IgE levels (ng/ml) of cat allergic (●) and non-allergic (○) individuals as measured directly *ex vivo* by ELISA. Bars represent medians. Statistical analysis was determined by Mann-Whitney U test of significance.

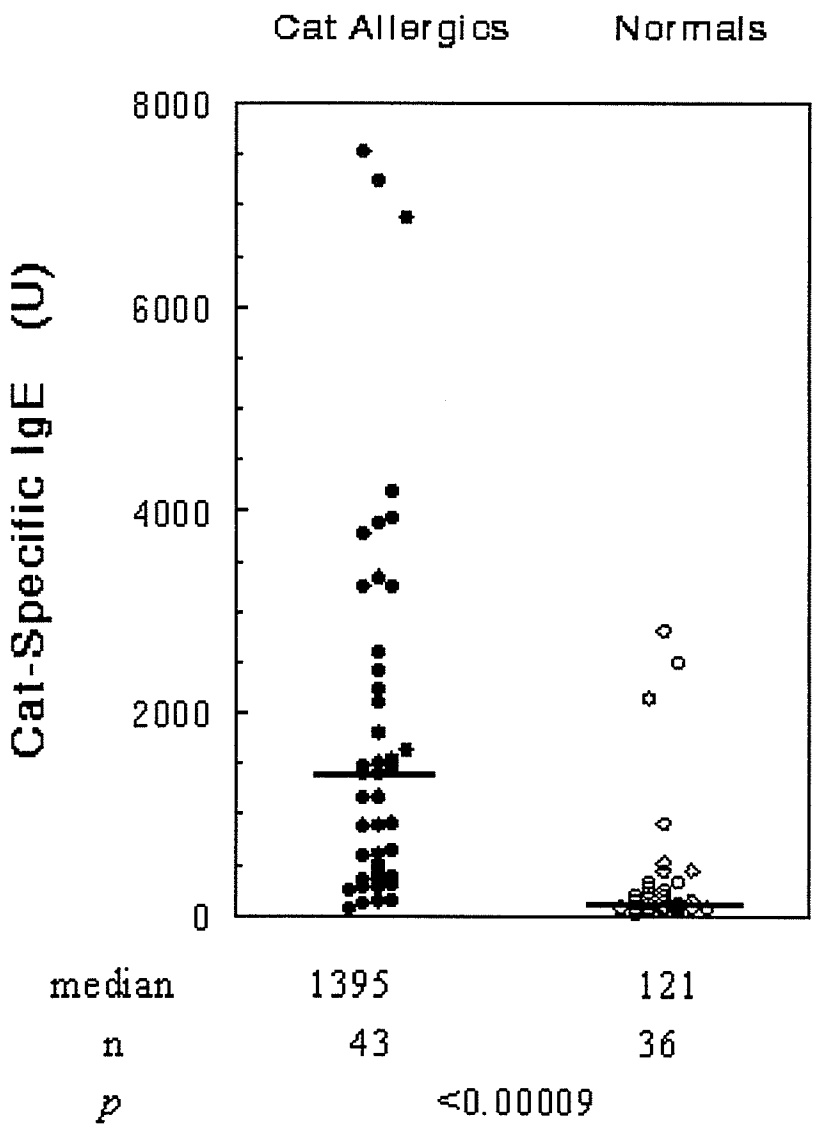

Figure 3. Cat antigen-specific IgE levels (arbitrary scale) of cat allergic (●) and non-allergic (○) individuals as measured directly *ex vivo* by ELISA. Bars represent medians. Statistical analysis was determined by Mann-Whitney U test of significance.

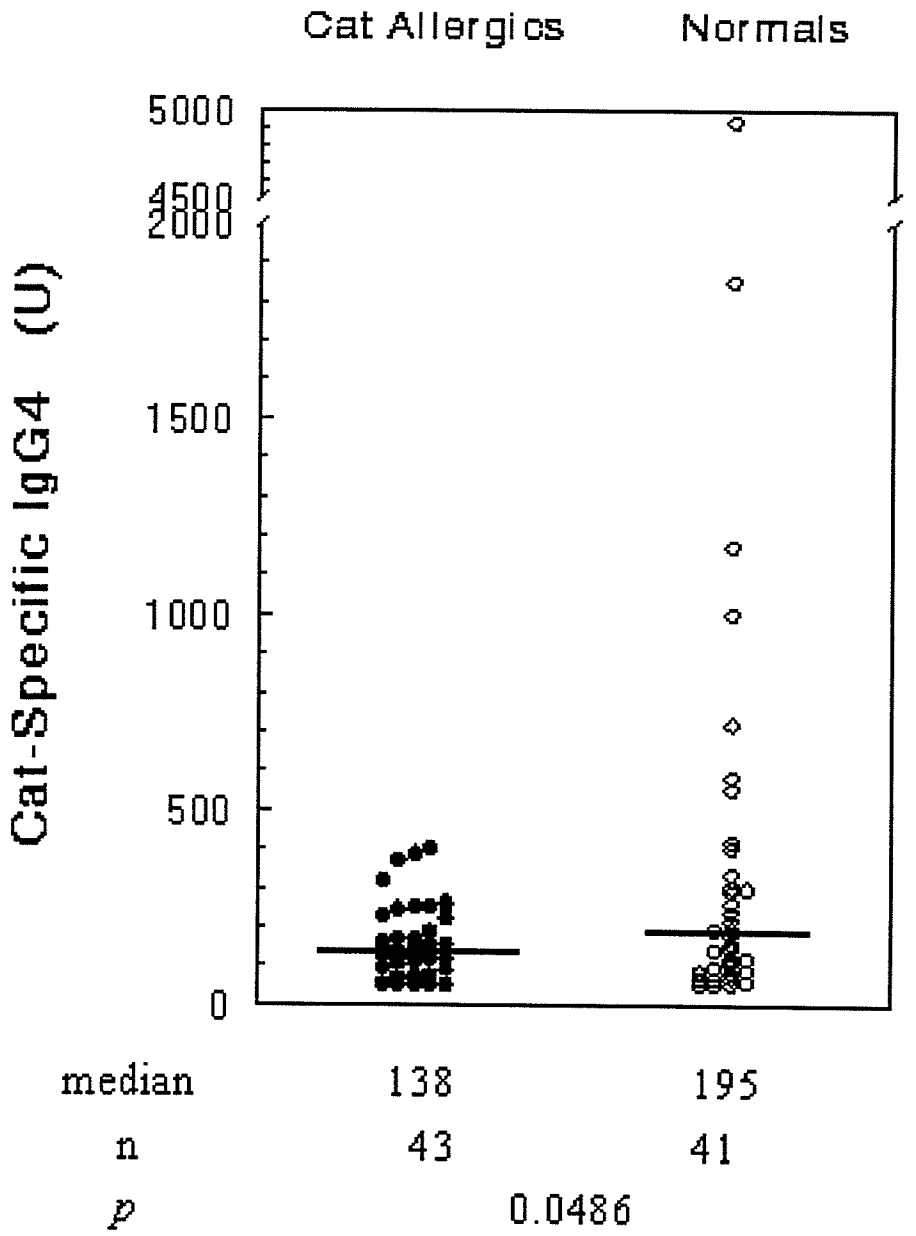

Figure 4. Cat antigen-specific IgG4 levels (arbitrary scale) of cat allergic (●) and non-allergic (○) individuals as measured directly *ex vivo* by ELISA. Bars represent medians. Statistical analysis was determined by Mann-Whitney U test of significance.

Cytokines

There are several advantages to using antigen, as opposed to polyclonal activators such as PHA or anti-CD3, to stimulate the cells for measurement of cytokine. One is that only the cells specific for the antigen respond to antigen, whereas all cells respond to polyclonal activators. Another is that the receptor through which activation is signalled by antigen is the same as the one activated *in vivo* by allergen exposure, whereas different receptors are responsible for activation signalling by polyclonal activators. When different receptors are used for activation the result can be qualitatively and quantitatively different. The difference in results obtained by antigen stimulation and polyclonal activation was shown by Imada et al. in a study of grass pollen allergic and non-allergic subjects (Imada 1995). With polyclonal activation, the response elicited from the normal and atopic groups was the same, yet that response was different for different polyclonal activators. PHA elicited a Th2-like response from both groups, while anti-CD3 elicited a Th1 response from both groups. However, stimulation with grass pollen elicited a Th1-like response from the non-allergic individuals and a Th2 response from allergic subjects. These results underline the importance of using antigen stimulation.

In this study, we also stimulated a subset of cells with 1% PHA as a comparison to the responses obtained with antigen. The IFNγ and IL-10 were measured by ELISA as described in the Materials and Methods section. The data are shown in Fig. 5 and 6 below. These IFNγ values are higher than those produced in response to antigen, however the IL-10 response was weaker. We found no difference in the levels of IFNγ produced by normal and allergic subjects, but a significant difference was seen in the IL-10 production,

PHA-induced IFNγ Production (U/ml) by Allergic and Non-Allergic PBMC

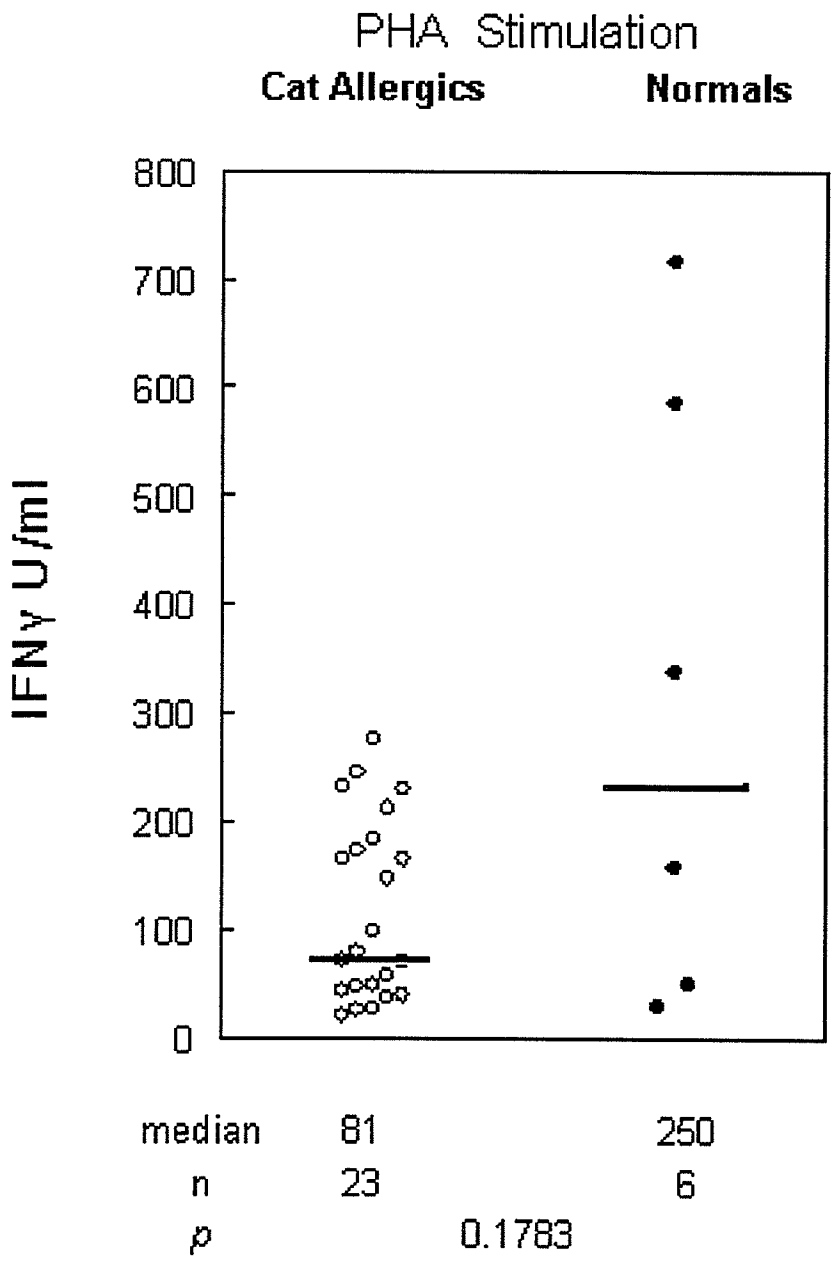

Figure 5. IFNγ production (U/ml) of cat allergic (●) and non-allergic (○) individuals as measured by ELISA after 2 days of culture with 1% PHA. Bars represent medians. Statistical analysis was determined by Mann-Whitney U test of significance.

PHA-induced IL-10 Production (pg/ml) by Allergic and Non-Allergic PBMC

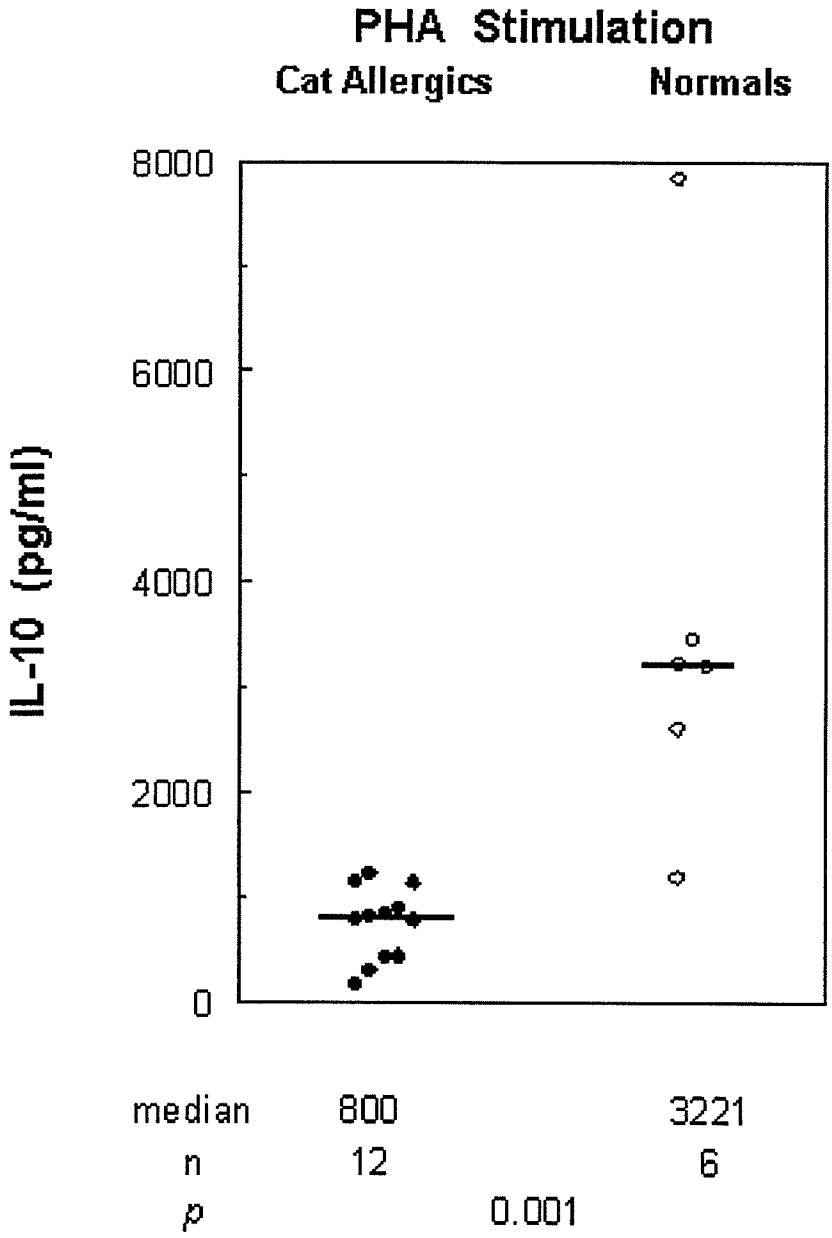

Figure 6. IL-10 production (pg/ml) of cat allergic (●) and non-allergic (○) individuals as measured by ELISA after 2 days of culture with 1% PHA. Bars represent medians.Statistical analysis was determined by Mann-Whitney U test of significance.

Cat antigen-induced IFNγ Production (U/ml) by Allergic and Non-Allergic PBMC

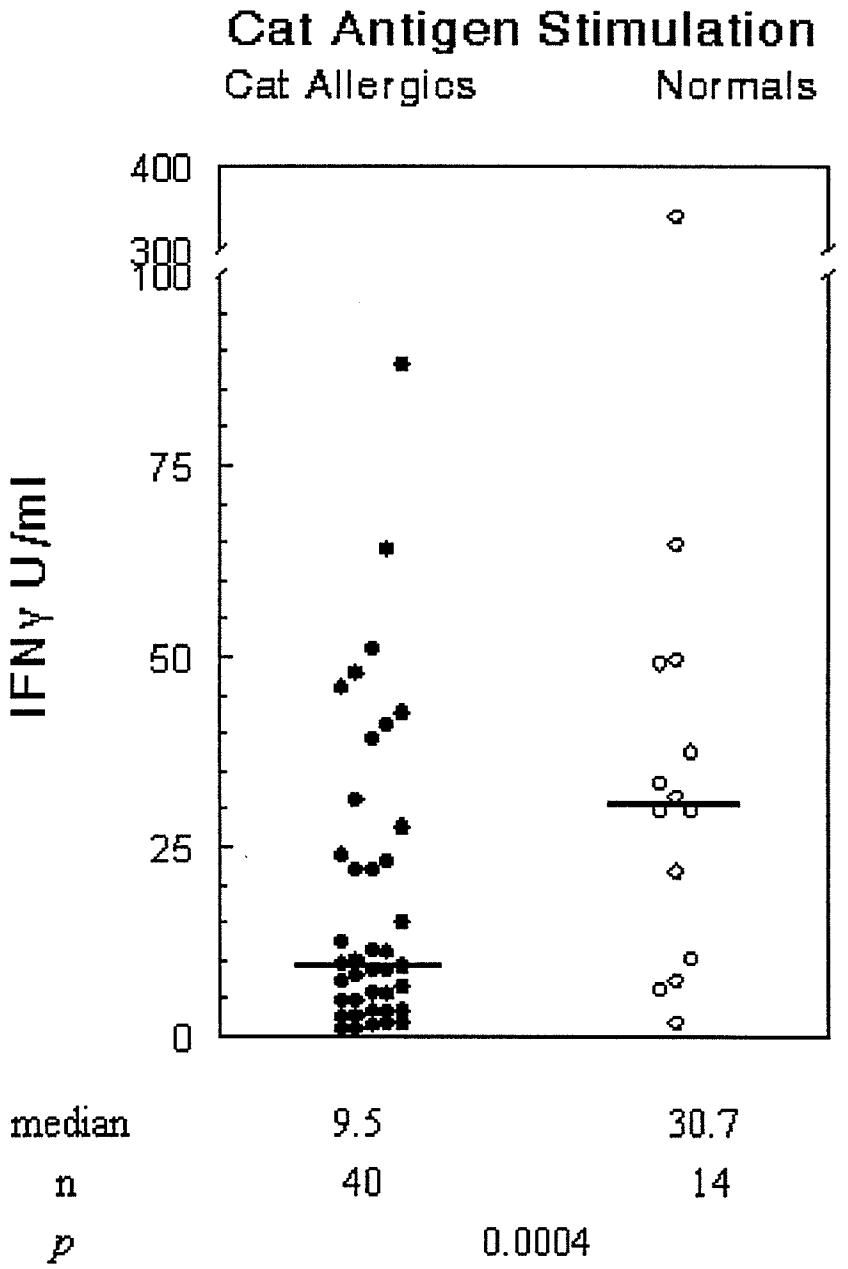

Figure 7. IFNγ production (U/ml) of cat allergic (●) and non-allergic (○) individuals as measured by ELISA after 4 days of culture with 500 BAU/ml (26.5 μg/ml) standardized cat hair extract (ALK) as the stimulating agent. Bars represent medians. Statistical analysis was determined by Mann-Whitney U test of significance.

Cat antigen-induced IL-10 Production (pg/ml) by Allergic and Non-Allergic PBMC

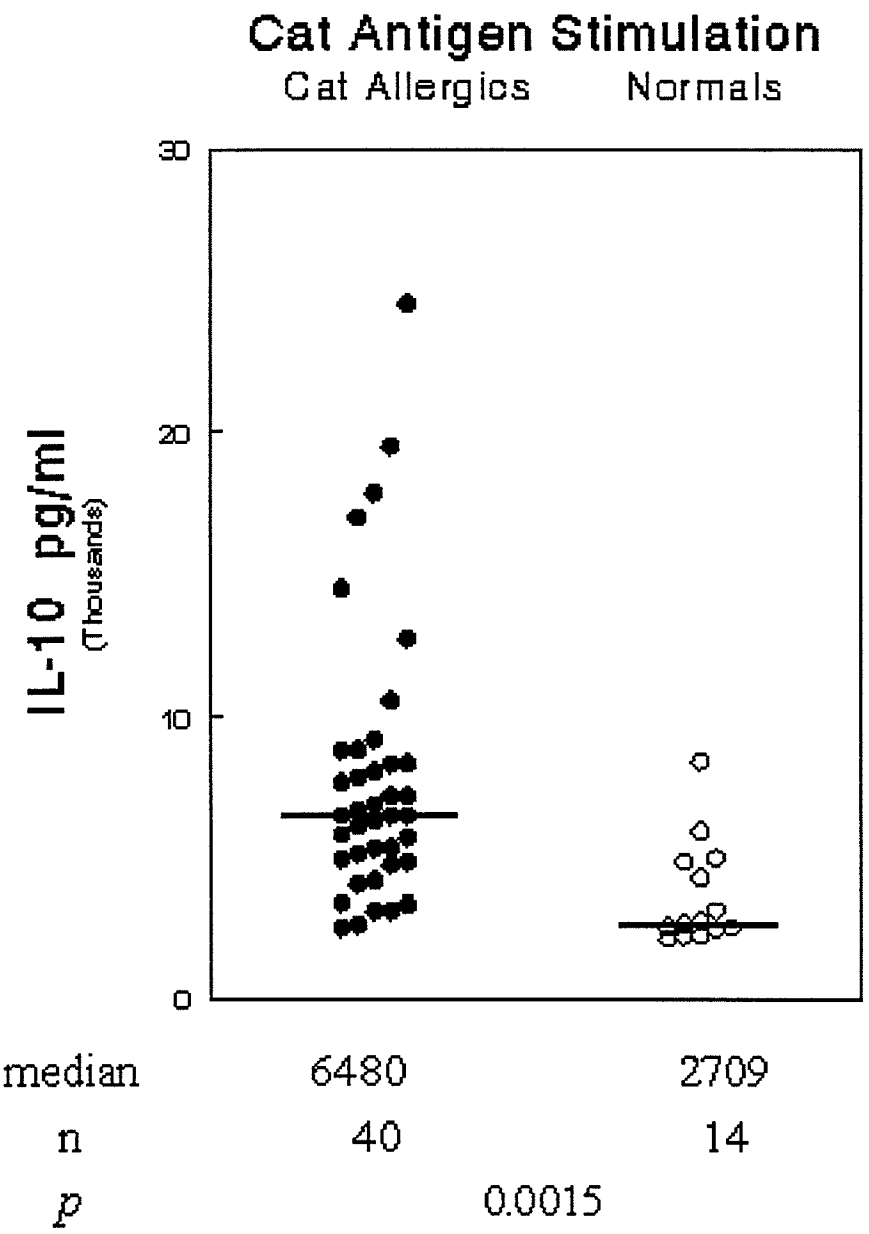

Figure 8. IL-10 production (pg/ml) of cat allergic (●) and non-allergic (○) individuals as measured by ELISA after 2 days of culture with 500 BAU/ml (26.5 µg/ml) standardized cat hair extract (ALK) as the stimulating agent. Bars represent medians. Statistical analysis was determined by Mann-Whitney U test of significance.

Cat antigen-induced IL-4 Production (pg/ml) by Allergic and Non-Allergic PBMC

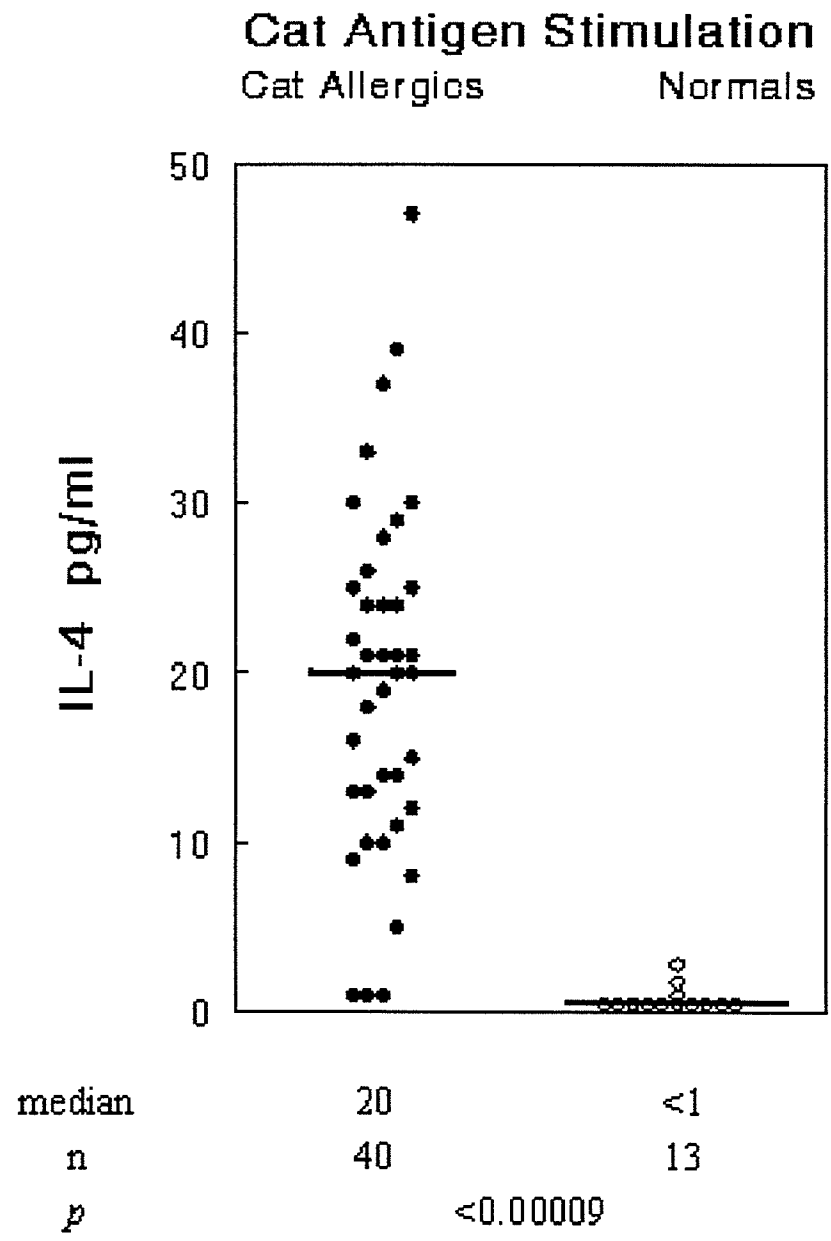

Figure 9. IL-4 production (pg/ml) of cat allergic (●) and non-allergic (○) individuals as measured by CT.h4S bioassay after 3 days of culture with 500 BAU/ml (26.5 μg/ml) standardized cat hair extract (ALK) as the stimulating agent. Bars represent medians. Statistical analysis was determined by Mann-Whitney U test of significance.

with the non-allergic group displaying a higher IL-10 response.

Very different results were obtained with allergen stimulation. PBMC were cultured with 500 BAU/ml and subsequently IFNγ, IL-10 and IL-4 were measured. The data are shown in Fig. 7, 8 and 9. The allergen-driven IFNγ was significantly higher in the non-atopic group, while IL-10 and IL-4 were both significantly higher in the atopic group. The data range is large and again, as seen with antibody levels, it is not normally distributed as determined by Kurtosis and Skewness test. The bars in these graphs are also medians. Again, there is considerable overlapping between the two groups, yet they are significantly different according to the non-parametric Mann Whitney U test.

Blocking Antigen Presentation

In one attempt to confirm that the activation and cytokine production induced by the cat antigen preparation is indeed antigen mediated, chloroquine was added with antigen or independently to PHA in culture. Chloroquine inhibits lysosomal enzymes (Matsuzawa 1980) and vesicle fusion process (Gonzalez-Noriega 1980). It also inhibits IL-2 production by T cell clones and interferes with T cell responsiveness to IL-2 (Landewé 1995). This explains why it has inhibited the IFNγ response of T cell clones to polyclonal activation (OKT3) (Landewé 1992). Tables 1 and 2 depict the effects of chloroquine on the IFNγ and IL-10 responses of normal PBMC to cat antigen and to PHA. The data indicate that chloroquine inhibits cat antigen-induced IFNγ and IL-10 in a dose-dependent manner.

Interestingly, chloroquine reduces the polyclonally activated IFNγ and IL-10 in a dose dependent manner as well. This is consistent with a requirement for antigen

Table 1. Effect of chloroquine on allergen induced IFNγ and IL-10 production by non-allergic subjects.

		in vitro stimulation conditions		
Subject Identifier	Cytokine	**Ag**	**Ag** + 20 μM chloroquine	**Ag** + 100 μM chloroquine
5	IFN	49.1	12.1	93.9
	IL-10	5193	5181	3219
7	IFN	29.8	1.5	2.0
	IL-10	5949	5710	1575
18	IFN	7.3	<0.6	1.5
	IL-10	2545	1953	590
19	IFN	29.8	1.2	<0.6
	IL-10	2817	2826	1553
21	IFN	64.7	0.6	21.6
	IL-10	6574	4727	1530
22	IFN	21.2	3.7	1.1
	IL-10	2268	2058	821

Chloroquine inhibition of IFNγ production (U/ml) and IL-10 production (pg/ml) by human PBMC of non-allergic subjects stimulated with 500 BAU/ml standardized natural cat antigen. Chloroquine concentrations of 0, 20 μM, or 100 μM were present for the duration of the culture. IL-10 was measured by ELISA 2 days after initiation of culture. IFNγ was measured by ELISA 4 days after initiation of the culture. Duplicate cultures performed.

Table 2. Effect of chloroquine on polyclonally activated IFNγ and IL-10 production by non-allergic subjects.

		in vitro stimulation conditions		
Subject Identifier	Cytokine	**PHA** 1%	**PHA** + 20 μM chloroquine	**PHA** + 100 μM chloroquine
5	IFN	159.2	86.8	35.2
	IL-10	3233	2120	1254
7	IFN	50.8	16.4	10.3
	IL-10	2610	2114	1823
18	IFN	31.1	4.8	1.2
	IL-10	3210	1092	551
19	IFN	339	82.6	2.4
	IL-10	3474	831	69
21	IFN	717	211	52.6
	IL-10	7830	4270	570
22	IFN	585	145.5	6.7
	IL-10	1195	274	<16

Chloroquine inhibition of IFNγ production (U/ml) and IL-10 production (pg/ml) by human PBMC of non-allergic subjects stimulated with 1% PHA. Chloroquine concentrations used at 0, 20 μM, or 100 μM for the duration of the culture. IL-10 and IFNγ were measured by ELISA 2 days after initiation of culture. Duplicate cultures performed.

processing and presentation in the activation of the majority of IFNγ synthesis. IL-10 production, while substantially inhibited in the presence of chloroquine remains substantial even at concentrations of chloroquine that block >90% of IFNγ synthesis. We speculate that this substantial residual IL-10 synthesis may reflect antigen processing independent sources (ie. monocytes) of IL-10. Further experiments are required to definitively test this hypothesis. The decreased IFNγ response to PHA in the presence of chloroquine is presumably due to interference with T cell responsiveness to IL-2, but the decreased IFNγ response to cat antigen in the presence of chloroquine could be due to blockage of antigen presentation or interference with T cell responsiveness.

As an independent approach to establishing the requirements for antigen processing and presentation via the exogenous pathway (as is required for soluble protein antigens such as Fel d 1) we utilized a cocktail of mouse monoclonal antibodies specific for human MHC class II monomorphic determinants with cat antigen in culture. This method should theoretically block presentation of exogenous antigen by APC to T cells. In practice, it completely blocked the IFNγ response by non-allergic subjects when added to culture at a concentration of 18 μg/ml, but it did not affect the IL-10 response. These results are shown in Table 3. These data support the speculation described immediately above about the relative contribution of cat antigen-specific T cells to IFNγ versus IL-10 responses. IFNγ and IL-10 have different cellular sources which may explain the fact that one was blocked, but the other was only partially (Tables 1-3). IFNγ is produced by T cells and NK cells, whereas IL-10 is produced by T cells but also by B cells, macrophages, and keratinocytes. The most notable difference appears to be that IL-10 is produced by

APC, which are the targets of this antibody. It is possible that the antibody has stimulated the APC to which it binds, resulting in IL-10 production yet blocking antigen presentation to T cells and therefore IFNγ production as well. Further experiments would be required to test this hypothesis rigorously.

At the same time, a mouse monoclonal antibody specific for human MHC class I was also tested in culture with cat antigen. It theoretically should not block the response because MHC class I is involved in the endogenous antigen presentation pathway, but not the exogenous antigen presentation pathway. However, it likewise completely blocked IFNγ, when added to culture at a concentration of 50 μg/ml while not affecting IL-10. These results are also shown in Table 3. Again, this antibody may have stimulated the APC to which it binds (a subset of its targets) resulting in IL-10 production. However, it is difficult to explain the loss of IFNγ production.

We speculated that one reason underlying the capacity of the anti Class I monoclonal antibody to interfere with cytokine synthesis could be non-specific cytotoxicity. Of particular concern was the HAT supplement to the media, which was used to grow the hybridoma source of this antibody, and could have caused release of a toxic substance. Therefore the antibody preparation was dialysed against serum-free RPMI and used in culture with human PBMC again (3 subjects) with no difference from the previous results. Moreover, a dialysed preparation of media containing HAT was cultured with murine spleen cells from OVA (alum) immunized mice and recall ovalbumin antigen.Addition of this media had no effect on IFNγ production. In the same experiment a dialysed irrelevant antibody preparation produced by a hybridoma grown in media

Table 3. Effects of anti MHC Class II and anti MHC Class I on IFNγ production (U/ml) and IL-10 production (pg/ml) in response to cat antigen (500 BAU/ml).

		in vitro stimulation conditions		
Subject Identifier	Cytokine	Ag	Ag + anti MHC Class II	Ag + anti MHC Class I
4	IFNγ	1.8	<1.2	<1.2
	IL-10	1487	541	1850
5	IFNγ	49.1	<0.6	<0.6
	IL-10	5193	9510	10753
7	IFNγ	29.8	<0.6	<0.6
	IL-10	5949	7550	8555
8	IFNγ	1.3	<1.2	<1.2
	IL-10	1974	4226	4214
18	IFNγ	7.3	<0.6	<0.6
	IL-10	2545	3640	5710
19	IFNγ	29.8	<0.6	<0.6
	IL-10	2817	4516	7410
21	IFNγ	64.7	<0.6	<0.6
	IL-10	6574	10700	13200
22	IFNγ	21.2	<0.6	<0.6
	IL-10	2268	4143	7546
32	IFNγ	2.6	<1.2	<1.2
	IL-10	4600	5066	6896
33	IFNγ	2.3	<1.2	<1.2

	IL-10	3337	4595	5475
34	IFNγ	5.2	14.4	not done
	IL-10	8726	142	not done
35	IFNγ	6.5	1.5	<1.2
	IL-10	1812	609	2023
36	IFNγ	1.3	<1.2	<1.2
	IL-10	25400	21700	28900
37	IFNγ	<1.2	<1.2	<1.2
	IL-10	6823	5458	6684

Inhibition of IFNγ production (U/ml) but not IL-10 production (pg/ml) by human PBMC of non-allergic subjects stimulated with 500 BAU/ml standardized natural cat antigen in the presence of antibodies against human MHC (anti human MHC class II used at 18 μg/ml; anti human MHC Class I used at 50 μg/ml). IL-10 was measured by ELISA 2 days after initiation of culture. IFNγ was measured by ELISA 4 days after initiation of the culture. Duplicate cultures performed.

Table 4. IFNγ production (U/ml) by murine spleen cells cultured with ovalbumin (OVA) recall antigen and various antibody preparations.

Culture Conditions	IFNγ Production (U/ml) in triplicate		
OVA + medium alone		11.5	19.6
OVA + media/HAT	14.0	16.8	20.3
OVA + 2682/HAT (anti-DNP; mouse IgE)	12.0	10.2	12.6
OVA + anti human class I	11.1	12.7	7.7
OVA + anti human class II	<0.5	<0.5	<0.5

Note: - indicates undetectable

containing HAT (HB 2682/HAT; mouse IgE with specificity for anti-DNP) did not affect the murine recall response to ovalbumin either. This argued that any residual HAT supplement itself was not non-specifically toxic, nor were any toxic substances released by the hybridoma cells as a result of culture with HAT. Furthermore, the anti MHC Class I antibody was also employed in this experiment and did not block the murine IFNγ production in response to ovalbumin indicating that this particular antibody preparation was not toxic. Interestingly, the anti human MHC Class II antibody blocked IFNγ production by the murine spleen cells indicating cross reactivity with mouse MHC. These results are shown in Table 4.

As a further control, in the same experiments that made use of the antibodies against MHC class I and class II, irrelevant antibodies were added to separate culture wells to control for the presence of antibodies and/or non-specific inhibition. The antibodies employed were (i) J11D, a rat IgM monoclonal antibody specific for mouse B cells, immature T cells, erythrocytes and neutrophils, the ligand for which is not expressed in humans; (ii) a mouse IgG1 monoclonal antibody against OVA; (iii) PK136 a mouse IgG2a antibody against mouse NK1.1, also not expressed in humans; and (iv) YTS169 a rat IgG2b antibody against mouse CD8 that does not react with human CD8. None of these antibodies consistently had an effect on cytokine production. Typical IFNγ production results of several allergic subjects stimulated with cat antigen and J11D and several non-allergic subjects stimulated with cat antigen and anti OVA are shown in comparison with identical cultures containing either no antibody or the anti MHC antibodies in Table 5. Antibodies can interfere with cat antigen driven cytokine

Table 5. Effects of antibodies with irrelevant specificities versus antibodies against the MHC on IFNγ (U/ml) responses to antigen.

	in vitro stimulation conditions			
Subject Identifier	Cat Antigen	anti MHC Class II	anti MHC Class I	Irrelevant specificity antibody
Allergic	500 BAU/ml	12.5 μg/ml	Not done	J11D
29	13.9	<1.2		11.5
31	43.7	11.9		22.5
42	37.7	17.3		1.4
Non-Allergic	500 BAU/ml	18 μg/ml	50 μg/ml	anti OVA 20 μg/ml
5	49.1	<0.6	<0.6	24.5
18	7.2	<0.6	<0.6	6.1
19	29.8	<0.6	<0.6	<0.6

Representative data from 6 allergic and 14 non-allergic subjects tested for IFNγ production (U/ml) in response to cat antigen (500 BAU/ml) in the presence or absence of MHC specific or irrelevant antibodies in culture at the indicated concentrations. IFNγ was measured by ELISA in day 4 bulk culture supernatants. Results shown are means of duplicate cultures.

production despite irrelevant specificities. However, none of these antibodies completely blocked IFNγ production the way the anti MHC antibodies did. In future, Fab dimers could be used instead so as to eliminate possible effects of the Fc portion of the antibody.

Thus, looking at the data as a whole, we see that the anti MHC Class I antibody, although not non-specifically toxic, has completely blocked IFNγ production in response to cat antigen. This argues that MHC Class I dependent pathways have some involvement in the exogenous antigen processing and presentation pathway. Particularly in light of some recent reports, which through other means have suggested a role for MHC Class I in exogenous antigen presentation by macrophages and mast cells (Harding 1994, Norbury 1995, Malaviya 1996) this finding should be pursued.

In summary, each of the antigen-specific response parameters measured was significantly different between the atopic and the non-atopic group. The atopic response was characterized by higher IL-4 and IL-10 production, lower IFNγ production, higher IgE levels (both cat specific IgE and total IgE) and lower cat specific IgG4 levels. This indicated a more Th2-like response to this antigen collection. Conversely, the response from the non-allergic group was more Th1-like, as it was characterized by more IFNγ and marginally elevated IgG4 as well as substantially lower IL-4, IL-10 and total and specific IgE synthesis. These data, and their statistical analysis is summarized in Table 6 below.

Table 6. Summary of Cat-Antigen-Induced Cytokine Production and Cat-Antigen-Specific Antibody Levels of Cat-Allergic and Non-Allergic Subjects.

Parameter	Allergic Median	Non-allergic Median	Mann-Whitney Significance
IL-4 (pg/ml)	20	<1	p <0.00009
IL-10 (pg/ml)	6480	2710	$p = 0.0015$
IFNγ (U/ml)	9.5	30.7	$p = 0.0004$
IgE (ng/ml)	116	28	$p < 0.00009$
cat-specific IgE	1395	121	$p < 0.00009$
cat-specific IgG4	138	195	$p = 0.0486$

Correlations Between Cytokine Production, Antibody Synthesis, and *in vivo* Sensitivity

Having characterized a number of putatively relevant immunologic parameters in these subjects, we next wished to evaluate the possible relationship in the whole study population between the antigen-driven cytokine production, antigen-specific antibody and clinical sensitivity as determined by skin test responses to the antigen. We evaluated the data with two-tailed Spearman bivariate correlations, because they are not limited to measuring linear fit and because the means were not normally distributed and therefore a least squares fit was inappropriate. It was important to establish what a reasonable expectation of a strong correlation between parameters was in terms of correlation coefficients and significance values in this diverse group of subjects. The outbred nature of the human race leads to infinite genetic differences between individuals. There is also the variability in lifestyle and environment which greatly affect the individual. The genetic and environmental exposure differences of the study subjects affect the strength of any correlations which may be tested. Therefore some more obvious correlations were tested first. A statistically significant correlation was observed between total IgE and cat-specific IgE with the correlation coefficient $r = 0.53$ and significance p value of 0.000. The graphical representation of this correlation is shown in Figure 10.

Another correlation which could be strongly suspected was that of antigen-specific IgE to the wheal intensity of the skin test response, because studies beginning at the turn of the century demonstrated that passive immunization with antigen-specific IgE

A Positive Correlation Between Cat-Specific IgE and Total IgE

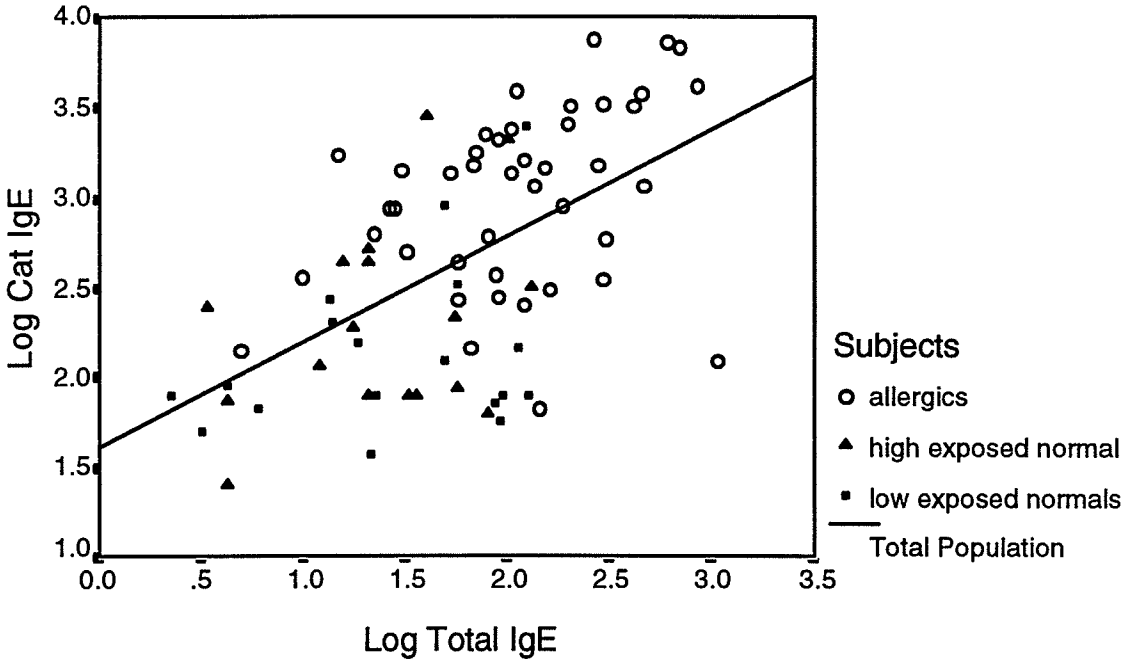

Figure 10. A statistically significant positive correlation between cat-specific IgE and total IgE both measured directly *ex vivo* by ELISA was found in the study population as a whole (n=80 subjects). A bivariate two-tailed Spearman correlation test determined that r = 0.53 $p = 0.000$.

A Positive Correlation Between Cat-Specific IgE and Wheal Intensity

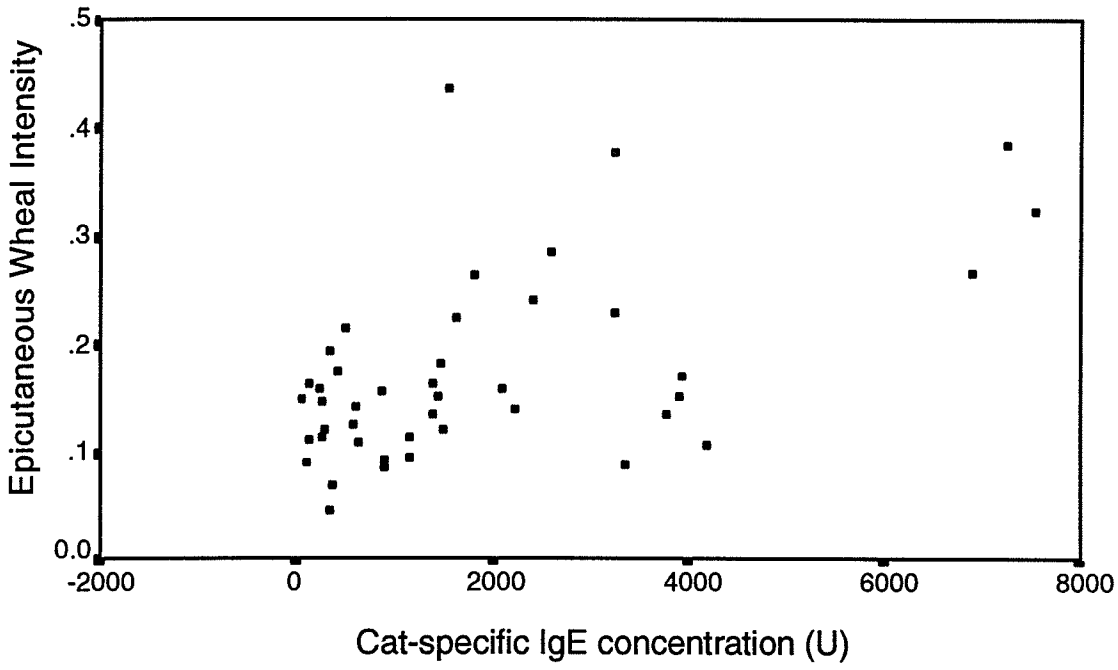

Figure 11. A statistically significant positive correlation between cat-specific IgE measured by ELISA directly *ex vivo* and wheal intensity in response to a cat antigen skin test was found in the allergic population (n=43 subjects). A bivariate two-tailed Spearman correlation test determined that $r = 0.43$ $p = 0.004$.

transferred the wheal response to antigen challenge to the recipient. As suspected, the correlation between cat-specific IgE and the wheal intensity was statistically significant. The r value was 0.4282 and the *p* value was 0.004. A graphical representation of the correlation is contained in Figure 11. This has established that specific IgE responses correlate to clinical sensitivity and, at the same time, that there is not a perfect linear relationship (r of 1.0) between the intensity of plasma IgE and intensity of skin test reactivity in any single subject. This strongly suggests that while specific IgE and clinical sensitivity are highly related in the population as a whole, other variables significantly affect the relationship between antibody (cat-specific) production and clinical hyperreactivity.

In contrast to specific IgE, total IgE did not correlate to the wheal intensity ($r = -0.09$, $p = 0.56$). This is shown in Figure 12. In this study there is clearly no relationship between total IgE and clinical sensitivity. These results underline the importance of measuring antigen-specific IgE as opposed to total IgE, which some other investigators have relied on previously.

A statistically significant positive correlation between cat antigen-induced *in vitro* IL-4 and cat-specific *ex vivo* IgE was observed ($r = 0.33$ $p = 0.017$). This is depicted in Fig.13. In contrast, cat antigen-induced *in vitro* IFNγ was statistically significantly negatively correlated to cat-specific IgE ($r = -0.33$ $p = 0.019$). Fig.14 depicts this correlation.

The positive correlation of *in vitro* antigen-induced IL-4 to antigen-specific IgE and the negative correlation of *in vitro* antigen-induced IFNγ to antigen-specific IgE is

Total IgE does not correlate to Wheal Intensity

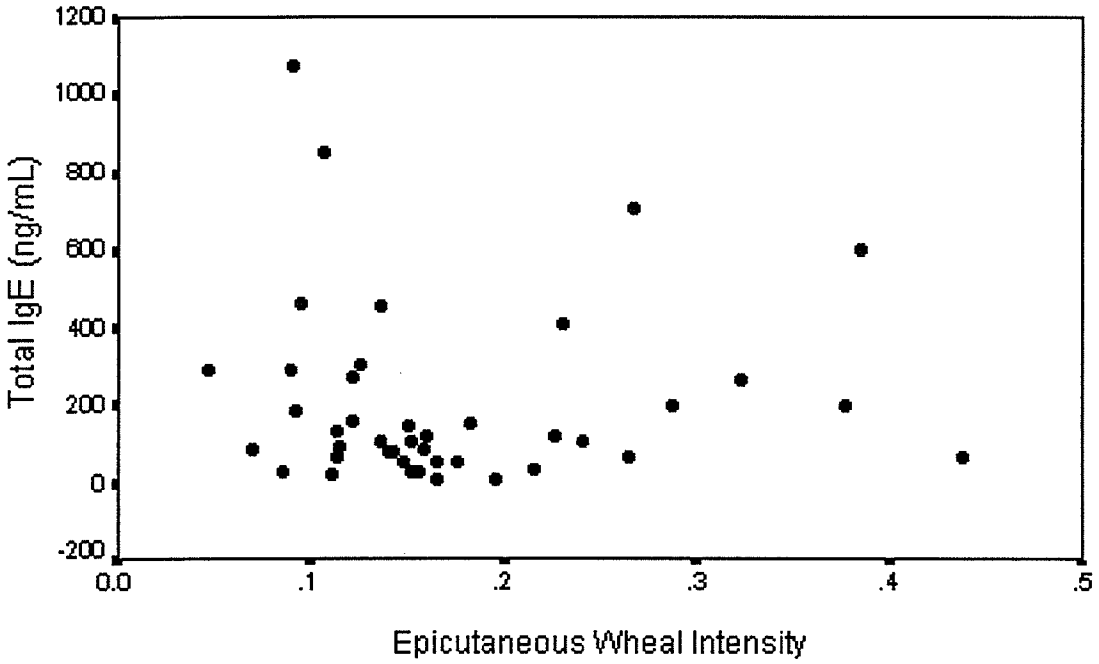

Figure12. No significant correlation was found between total IgE (ng/ml) measured directly *ex vivo* by ELISA and the epicutaneous wheal intensity (n=40 subjects). A bivariate two-tailed Spearman correlation test was used.

A Positive Correlation Between Cat-Specific IgE and IL-4

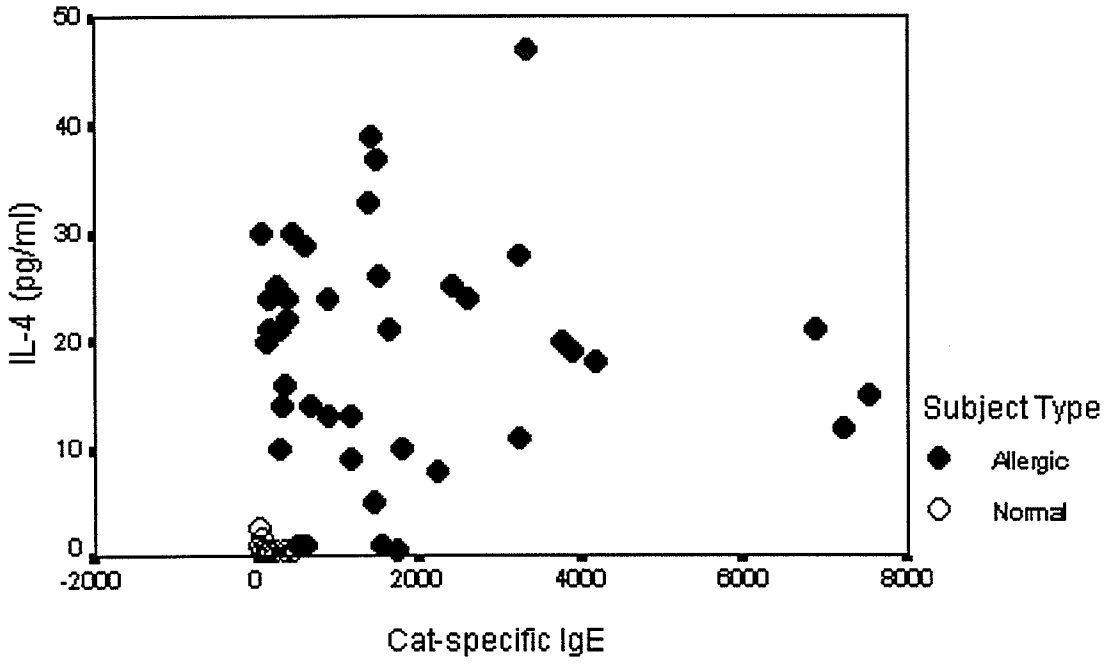

Figure 13. A statistically significant positive correlation between cat-specific IgE measured by ELISA directly *ex vivo* and *in vitro* IL-4 production in response to cat antigen stimulation (500 BAU/ml) was found in the study population as a whole (n=53 subjects). A bivariate two-tailed Spearman correlation test determined that r = 0.33 p = 0.017.

A Negative Correlation Between Cat-Specific IgE and IFNγ

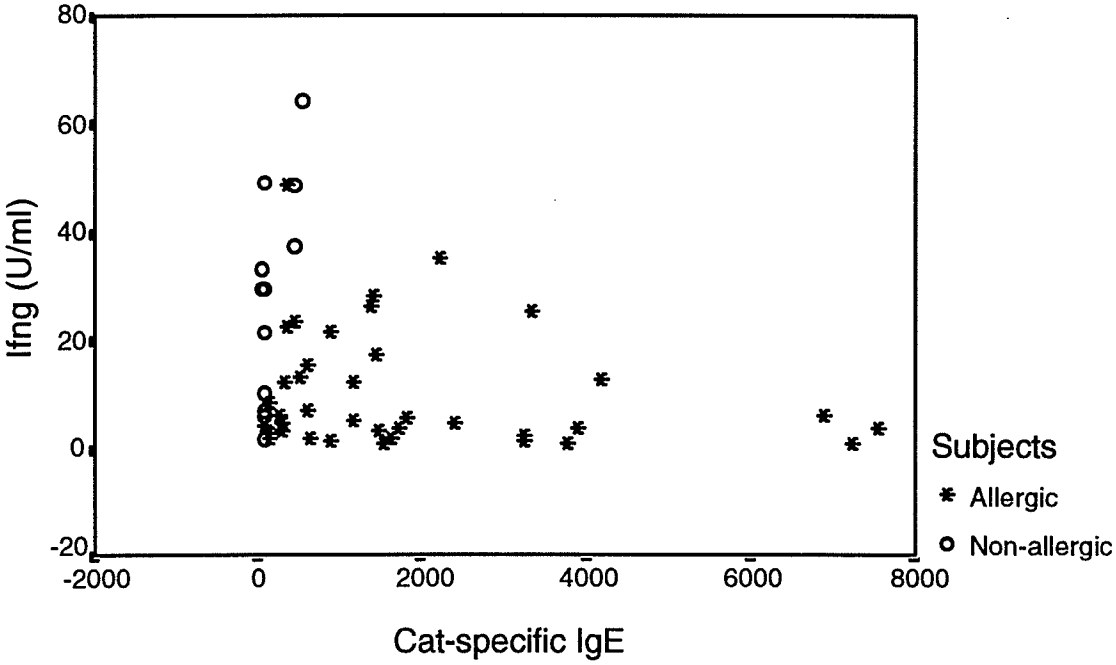

Figure 14. A statistically significant negative correlation between cat-specific IgE measured by ELISA directly *ex vivo* and *in vitro* IFNγ production in response to cat antigen stimulation (500 BAU/ml) was found in the study population as a whole (n=54). A bivariate two-tailed Spearman correlation test determined that r = -0.33 $p = 0.019$.

consistent with the hypothesis that Th2-like cytokine production is associated with elevated IgE production. Indeed, the ratio of IFNγ to IL-4 is significantly negatively correlated to cat-specific IgE ($r = -0.36$, $p = 0.017$). These findings, combined with the finding that cat-specific IgE is positively correlated to the wheal intensity, argue that antigen-driven IL-4 and IFNγ may be appropriate parameters for predicting clinical sensitivity.

Given that IL-10 is considered a Th2 cytokine in the murine system, but is not clearly Th1 or Th2 in the human system, as determined by evaluation of cytokine synthesis patterns of approximately 150-250 long term $CD4^+$T cell clones described in the literature, it was interesting to test whether IL-10, like IL-4, would positively correlate with specific IgE. No significant correlation was found between antigen-induced IL-10 production and specific IgE ($r = 0.21$ $p = 0.084$). However, IL-10 was found to be significantly correlated to IL-4 production ($r = 0.32$ $p = 0.021$) suggesting that, under the conditions tested, (short term antigen-driven primary culture of unselected PBMC populations rather than highly selected, long term $CD4^+$T cell clones), antigen-driven IL-4 and IL-10 production are coordinately regulated.

IFNγ production in response to antigen stimulation was not correlated (positively or negatively) to either antigen-induced IL-4 or IL-10 production. This is interesting considering that IFNγ responses suppress the development of IL-4 producing cells *in vitro* (reviewed in Paul 1994).

Given the controversy regarding the role of IgG4 in allergy it was interesting to test for a correlation between specific IgG4 and the wheal intensity. However, there was

no significant correlation between these parameters (r = -0.0661 $p = 0.673$). This finding supports neither the blocking antibody theory nor the anaphylactic antibody theory.

Given the existence of some common cytokine regulatory influences on the production of IgE and the IgG4 subclass, it was interesting to test the same antigen induced cytokines for correlations to specific IgG4, and compare them to those observed with specific IgE. It was shown in *in vitro* culture of human PBMC that IL-4 increases IgG4 production under the same conditions which are optimal for IgE production, and that this IL-4 mediated increase in IgG4 levels was suppressed by addition of recombinant IFNγ in most subjects (Sutherland 1993). However, they also concluded that additional signals are influential in determining which isotype production is promoted. In our study, in contrast to specific IgE, none of the cytokines measured significantly correlated to specific IgG4. This data is included in the summary in Table 7. These results would also argue that signals other than IL-4 and IFNγ are important in regulating IgG4.

Interestingly, specific IgG4 was weakly correlated to specific IgE (r = 0.2332 p = 0.039) over the population as a whole. In fact, the correlation was very strong in non-allergic individuals (r =0.7666 p <0.0005), while in allergic individuals there was not a significant correlation (r = 0.2959 $p = 0.051$). Figures 15 and 16 display these findings. The correlation of IgE to IgG4 has had only limited study to date. Peng et al. (1992) demonstrated a correlation of ragweed specific IgE to ragweed specific IgG4 in allergic individuals after two years of immunotherapy, but not before immunotherapy. The strong correlation between the specific IgE and specific IgG4 in this study in one group, but not the other, as well as the nearly distinct separation of the two groups in the graph (Fig. 15)

Cat-specific IgE strongly correlates to Cat-specific IgG4 in non-allergic subjects only.

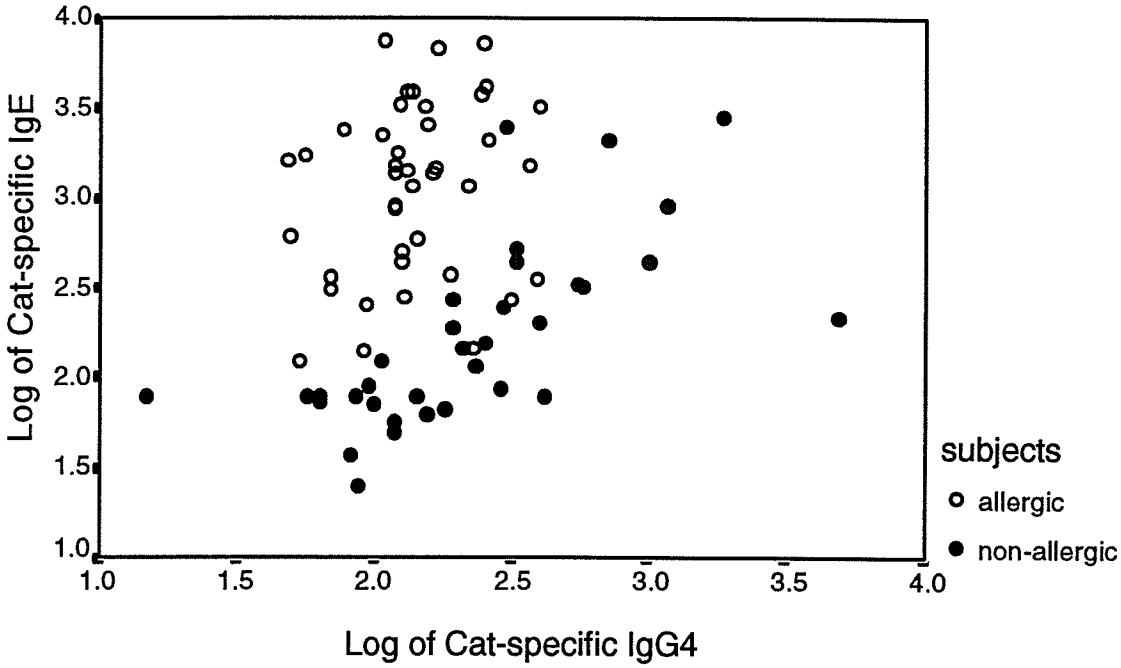

Figure 15. A statistically significant positive correlation was found between cat-specific IgE and cat-specific IgG4, both measured in plasma directly *ex vivo* by ELISA (n=80). A bivariate two-tailed Spearman correlation test determined that r = 0.23 $p = 0.39$. In the allergic individuals alone (○) there was not a significant correlation r = 0.29 $p = 0.051$, but in the non-allergic individuals (●) there was a strongly significant correlation r = 0.77 $p < 0.0005$.

Cat-specific IgE strongly correlates to Cat-specific IgG4 in non-allergic subjects only.

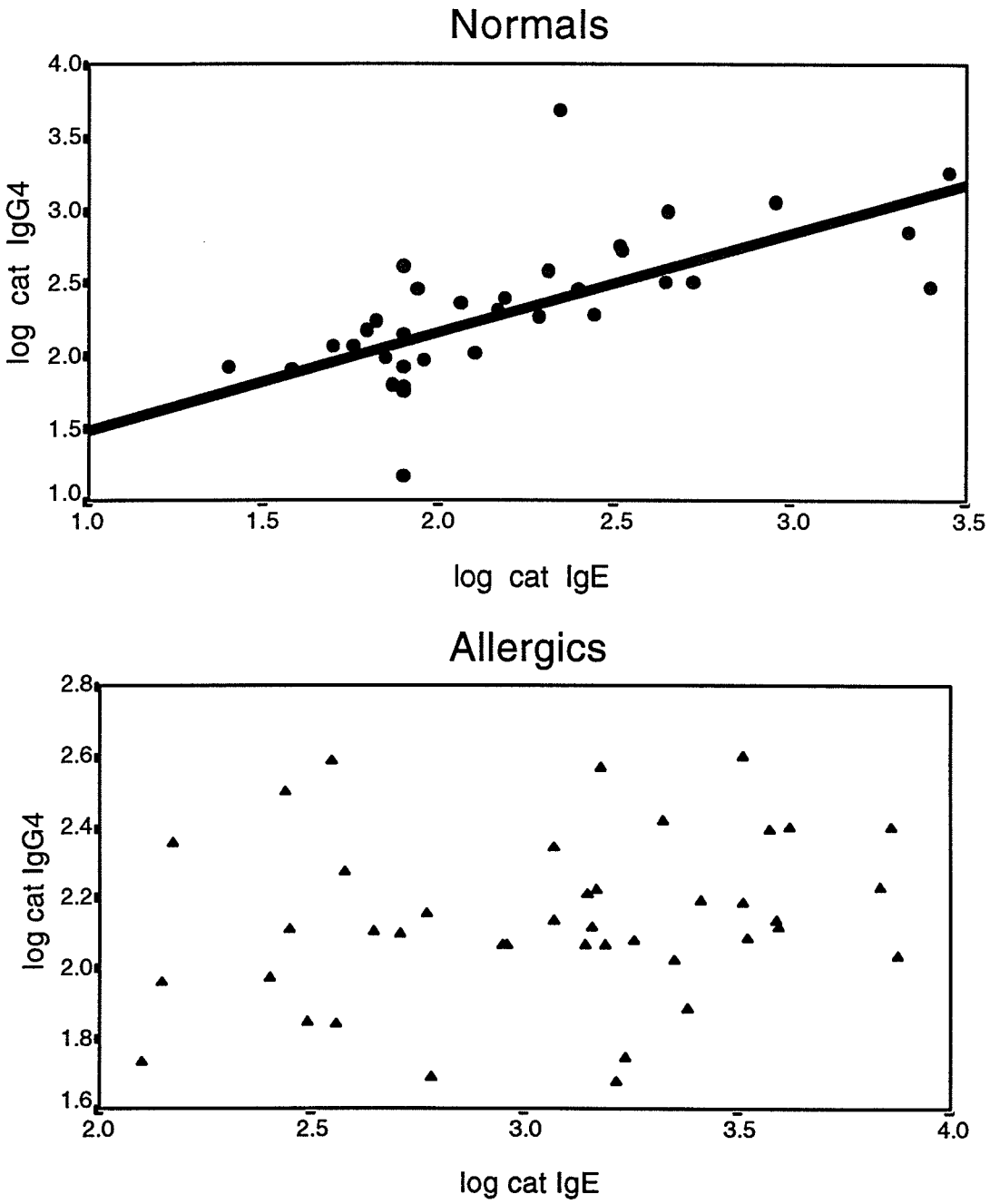

Figure 16. A strong statistically significant positive correlation was found between cat-specific IgE and cat-specific IgG4, both measured in plasma directly *ex vivo* by ELISA in the non-allergic individuals $r = 0.77$ $p < 0.0005$. In the allergic individuals there was not a significant correlation $r = 0.29$ $p = 0.51$.

indicated some importance for this relationship.

Selected Populations

The next stage in our analysis looked at selected subpopulations (ie. non-atopic versus cat allergic) rather than at the population as a whole. Within the allergic population alone there was not a significant correlation between the intensity of IL-4 synthesis and specific IgE ($r = -0.1033$ $p = 0.521$). Neither is there a significant correlation between IFNγ and specific IgE in this subgroup ($r = -0.16$ $p = 0.318$). Thus, among cat allergic subjects the intensity of *in vitro* IL-4 and IFNγ synthesis is not linearly related to the antigen specific IgE levels measured *ex vivo*. Thus, while the intensity of various cytokine or antibody responses discussed above correlate strongly with immediate hypersensitivity in the population as a whole (as approximated by the intensity of skin test reactivity), incremental differences in production of any given cytokine or antibody (ie. 30% increase, two-fold increase, etc.) fail to associate well with the intensity of the *in vivo* reaction. This suggests that while analysis of cytokine (or antibody) synthesis provides a good indicator of sensitivity on a population basis, identification of limited changes in a given individual may be of limited prospective value. This hypothesis remains to be tested explicitly and will require the availability of consistently effective means of altering *in vivo* sensitivity to allergen.

Table 7. Summary of Correlations of Wheal Intensity to Antibodies to Cytokines.

Correlation	Whole Population		Normal Population		Allergic Population	
	r	*p*	r	*p*	r	*p*
Wheal to IgE*					0.4282	0.004
Wheal to IgG4					-0.0661	0.673
IgE to IgG4	0.2332	0.039	0.7666	<0.0005	0.2959	0.051
IL-4 to IgE	0.4284	0.004			-0.1033	0.521
IFNγ to IgE	-0.3277	0.019			-0.16	0.318
IFNγ/IL-4 to IgE	-0.358	0.017			-0.1187	0.478
IL-10 to IgE	0.21	0.084				
IL-4 to IgG4	-0.19	0.169				
IFNγ to IgG4	0.1048	0.46				
IL-10 to IgG4	-0.0254	0.834				
IFNγ to IL-4	-0.07	0.644				
IFNγ to IL-10	-0.25	0.07				
IL-10 to IL-4	0.32	0.021				

* all IgE is specific IgE

IL-15

In an independent series of preliminary experiments, we examined the potential role of IL-15 in recall responses. IL-15 has been shown *in vitro* to augment proliferation, cytokine production and effector function of lymphoid cells such as B, T, and NK cells. Therefore, we hypothesized that this cytokine may play an important role in initiating or maintaining a variety of immune responses. Functions identified *in vitro* for IL-15 are similar to IL-2. This is not surprising given that they share some receptor signalling components. A comparison of the two cytokines follows in Table 8. We recently found that IL-2 augments the Th1 nature, but not the Th2 nature of a recall response. In a murine model, IL-2 was required for IFNγ but not IL-4 synthesis from antigen-primed T cells (Yang 1993). In a human model, IL-2 is not strictly required for, but does augment allergen-induced IFNγ synthesis while its presence or absence does not affect IL-4 synthesis (Imada et al. in preparation). Whether IL-15 is also required for, or capable of, augmenting exogenous antigen-specific Th1 or Th2 responses has not been examined and was one focus of our study.

Table 8. Comparison of the Properties of IL-2 and IL-15.

Properties	IL-2	IL-15
Functions:		
activate NK cell proliferation, cytokine production and cytotoxicity	yes	yes
costimulate B cell proliferation and Ig secretion	yes	yes
activate T cell proliferation and induce cytolytic effector cells	yes	yes
chemoattract T cells	yes	yes
augment Th1 recall response	yes	unknown
Cellular expression:	activated peripheral T	macrophage, epithelial, fibroblast
Tissue expression:		placenta, skeletal muscle, kidney, lung, heart, pancreas
Receptor:	IL-2Rα, IL-2Rβ, γ_c	IL-15Rα, IL-2Rβ, γ_c
Cellular Expression	macrophage, T, NK, B	stromal, macrophage, T, NK, B
Tissue Expression	spleen, lung, heart, muscle	liver, spleen, lung, heart muscle, kidney
Shed soluble receptor	yes	yes
Receptor affinity	10^{-8} M	10^{-11} M

IL-15 Stimulation of Non-Specific Cytokine Production

In our initial experiments we needed to determine the concentration of recombinant IL-15 that led to non-specific activation of cytokine synthesis. By way of example, recombinant IL-2 at high levels in culture (>50 U/ml) leads to substantial proliferation and cytokine synthesis (Lefkovits 1984). To examine the non-specific effect of IL-15 on spontaneous cytokine production we cultured PBMC *in vitro* in the presence of exogenous recombinant IL-15 (and the absence of cat antigen) and measured IFNγ and IL-10 production in the supernatants. The data are shown in Tables 9 and 10. We detected no significant increase in production of either cytokine by atopic or non-atopic subjects in the presence of IL-15 at doses between 10-1000 pg/ml. These results are in contrast to IL-12, another macrophage cytokine, which does induce IFNγ production from PBMC of non-atopic individuals at concentrations of 25 to 100 pg/ml. (Li et al. in preparation). These data demonstrate that addition of recombinant IL-15 at the concentrations tested does not lead to non-specific activation of IFNγ synthesis in an antigen independent manner.

IL-15 Stimulation of Antigen Induced Cytokine Production

The effect of supplementary IL-15 on Th1 and Th2-like cytokine production in response to antigen stimulation was examined in a similar manner. Standardized cat hair extract (ALK) at a concentration of 500 BAU (26.5 μg/ml total protein), which was shown to be the optimal concentration for cytokine production in this and other studies,

Table 9. Effect of IL-15 on non-specific **IFNγ (U/ml)** production by non-stimulated PBMC.

Subject Identifier	IL-15 (pg/ml)			
Non-allergic	0	10	100	1000
8	<0.6	nd	<0.6	6.7
9	<1.2	nd	<1.2	<1.2
14	<0.6	nd	0.7	0.7
19	0.8	nd	0.7	7.2
21	1.5	nd	1	1.6
34	<1.2	nd	<1.2	1.5
37	13.9	nd	11.3	13.6
39	1.4	nd	0.8	3.5
Allergic				
7	<0.6	1	3.6	4
8	<0.6	<0.6	<0.6	<0.6
30	<0.6	<0.6	<0.6	6.5

IFNγ production (U/ml) by human PBMC of non-allergic and cat-allergic subjects measured by ELISA after 4 days of culture with exogenous recombinant IL-15 at either 0, 100 or 1000 pg/ml. nd = not determined.

Table 10. Effect of IL-15 on non-specific **IL-10 (pg/ml)** production by unstimulated PBMC.

Subject Identifier	IL-15 (pg/ml)			
Non-allergic	0	10	100	1000
4	67	nd	65	92
8	349	nd	266	404
14	<32	nd	<32	<32
19	<32	nd	<32	<32
21	<32	nd	<32	<32
25	108	nd	154	443
29	170	nd	195	283
30	59	nd	56	104
31	75	nd	170	220
32	137	nd	135	178
33	50	nd	127	169
34	313	nd	264	280
37	<32	nd	<32	<32
Allergic				
7	29	49	66	16
8	363	496	493	517
17	192	170	211	296
30	305	326	442	622

IL-10 production (pg/ml) by human PBMC of non-allergic and cat-allergic subjects measured by ELISA after 2 days of culture with exogenous recombinant IL-15 at either 0, 100 or 1000 pg/ml. nd = not determined.

was used to stimulate PBMC in the presence or absence of 100-1000 pg/ml IL-15. It should be noted that these concentrations of IL-15 are 10 fold lower than those studied by Seder et al. (1995) to costimulate PBMC of HIV-infected and non-infected individuals with HIV-1 *env* synthetic peptides and IL-15 (Seder 1995). After incubation with IL-15, supernatants were harvested and analyzed for IFNγ (Th1), IL-5 (Th2) and IL-10. The data are shown in Tables 11, 12 and 13 respectively. It can be seen that addition of supplementary IL-15 substantially increases (approximately 10-fold) allergen-induced IFNγ production by atopic and non-atopic PBMC at 1000 pg/ml and in 8 of 12 subjects at 100 pg/ml (Table 11). However, in contrast, IL-15 does not affect allergen-induced IL-10 production by either atopic or non-atopic PBMC at either of these doses. Under the conditions tested IL-15 is not capable of detectable effects on IL-5 production. As such the data suggest that IL-15 may support or promote the specific Th1 associated cytokine response without boosting or suppressing the specific Th2 associated cytokine response.

Relationship Between IL-15 and IL-2 in Promoting Th1 Responses

To test whether IL-15 required endogenous IL-2 to induce IFNγ synthesis from normal PBMC stimulated with allergen, PBMC were cultured with IL-15 and anti-IL-2 simultaneously in the presence of cat allergen. The results (shown in Table 14 below) indicate that IL-15 does increase allergen-induced IFNγ in the absence of active IL-2. In the presence of anti IL-2 most subjects responded to 100 pg/ml IL-15 and all responded to 1000 pg/ml (compare columns 5 and 6 to column 4). In fact, at 100 or 1000 pg/ml IL-15 some subjects produced the same levels of IFNγ whether the anti IL-2 antibody was

Table 11. Effect of IL-15 on allergen-induced **IFNγ (U/ml)** production.

Subject Identifier	IL-15 (pg/ml)			
Non-allergic	0	10	100	1000
4	1.8	nd	10.8	19
8	340	nd	597	1816
9	1.7	nd	2.6	24
14	6.2	nd	60	421
19	1.3	nd	1.6	1
21	26.6	nd	52.1	226.4
34	10.2	nd	22.3	163
37	31.6	nd	31	94.2
39	37.6	nd	39.7	137
Allergic				
7	<0.6	<0.6	3.3	5.7
17	0.7	0.9	3	11.4
30	1.3	2.1	6.6	16.2

IFNγ production (U/ml) by human PBMC of non-allergic and cat-allergic subjects measured by ELISA after 4 days of culture with natural standardized cat antigen (500 BAU/ml) exogenous recombinant IL-15 at either 0, 100 or 1000 pg/ml. nd = not determined.

Table 12. Effect of IL-15 on allergen-induced **IL-5 (pg/ml)** production.

Subject Identifier	IL-15 (pg/ml)	
Non-allergic	0	1000
8	<16	26.1, 156.1
9	<16	<16
14	<16	<16
21	<16	<16
34	<16	<16
19	<16	nd
37	nd	41.6
39	<16	nd

IL-5 production (pg/ml) by human PBMC of non-allergic subjects measured by ELISA after 2 days of culture with natural standardized cat antigen (500 BAU/ml) and exogenous recombinant IL-15 at either 0, 100 or 1000 pg/ml. nd = not determined.

Table 13. Effect of IL-15 on allergen induced **IL-10 (pg/ml)** production.

Subject Identifier	IL-15 (pg/ml)			
Non-allergic	0	10	100	1000
4	1487	nd	1349	1378
8	1974	nd	1632	1969
9	2644	nd	2623	2564
14	2567	nd	2274	1293
19	2152	nd	2737	<32
21	8375	nd	7641	7979
34	2213	nd	2720	1767
37	2485	nd	2297	2626
39	2773	nd	2939	3123
Allergic				
8	2410	nd	2450	nd
17	2729	2799	3056	2319
30	2966	2409	2369	2149

IL-10 production (pg/ml) by human PBMC of non-allergic and cat-allergic subjects measured by ELISA after 2 days of culture with natural standardized cat antigen (500 BAU/ml) and exogenous recombinant IL-15 at either 0, 100 or 1000 pg/ml. nd = not determined.

Table 14. IFNγ production (U/ml) by human PBMC of non-allergic subjects measured by ELISA after 4 days of culture with natural standardized cat antigen (500 BAU/ml) and exogenous recombinant IL-15 at either 0, 100 or 1000 pg/ml and anti IL-2 antibody 1/2500. nd = not determined.

Table 14. IL-15 can act independently of IL-2.

	1	2	3	4	5	6
IL-15 (pg/ml)	0	100	1000	0	100	1000
anti IL-2	0	0	0	0.0004	0.0004	0.0004
Subject Identifier	IFNγ					
8	340	597	1816	346	650	992
9	1.7	2.6	24	0.8	3.4	23.5
14	6.2	60	421	7.4	76.9	399
19	1.3	1.6	1	0.9	2.6	38
21	26.6	52.1	226.4	12.7	44.7	225
34	10.2	22.3	163	5.4	6.9	139.2
37	31.6	31	94.2	7.6	14.5	36.4
39	37.6	39.7	137	10.4	14	124.5
4	1.8	10.8	19	1.2	7	19.5
12	7	nd	nd	3.6	32.2	122.4
26	<1.2	nd	nd	<1.2	2.8	8.4
29	1.9	nd	nd	<1.2	2.9	10.7
31	<1.2	nd	nd	<1.2	1.2	5.9
32	2.6	nd	nd	3	10.7	65.9
33	2.3	nd	nd	1.4	4.5	20.4
16	4.1	nd	nd	1.4	2.2	3.6
23	<1.2	nd	nd	<1.2	5.6	16
25	<1.2	nd	nd	1.1	2.1	13.5
30	<1.2	nd	nd	<1.2	<1.2	1.9
35	6.5	nd	nd	6.1	15.4	66.4
36	1.3	nd	nd	1.4	3.3	16.9

present or not (compare column 6 to column 3 and column 5 to column 2) . This raises an interesting question. Can IL-15 substitute for IL-2? Other subjects produced less IFNγ in response to antigen and IL-15 costimulation when the anti IL-2 antibody was present, however this level of IFNγ was still substantially higher in most cases than if antigen was used for stimulation without IL-15 in the presence of anti IL-2. This finding raises the question of whether IL-15 and IL-2 can synergize. Further experiments will be required to address this question definitively.

As an alternative method of observing IL-15 effects on cytokine production in the absence of IL-2, we cultured PBMC of 3 subjects with antigen and IL-15 and an anti IL-2Rα antibody of rabbit origin called 2A3. This approach differs from that described above in that the first approach attempts to neutralize soluble IL-2 whereas the second approach blocks the interaction of IL-2 with the high affinity IL-2R. Recombinant IL-15 in the absence of anti IL-2R augmented antigen-induced IFNγ production as seen before, however, in the presence of anti IL-2Rα IL-15 had no effect. This result may have been affected by steric hindrance of IL-15 or cross reactivity of the antibody to IL-15Rα.

To test whether IL-15 and IL-2 could synergize in inducing increased IFNγ production, PBMC of 8 non-allergic individuals were cultured with both IL-15 and IL-2 in the presence of cat allergen. The data follows in Table 15. IL-15 or IL-2 alone each induced IFNγ production from allergen stimulated normal PBMC, and, in all but one subject, they did synergize in this regard at the above concentrations. For example subject 9 produced 1.7 U/ml IFNγ in the absence of exogenous cytokine, 4.9 U/ml in the presence of 10 U/ml IL-2, 24 U/ml in the presence of 1000 pg/ml IL-15 and 121.2 U/ml IFNγ in

Table 15. IL-15 synergizes with IL-2.

	1	2	3	4	5	6
IL-15 (pg/ml)	0	100	1000	0	100	1000
IL-2 (U/ml)	0	0	0	10	10	10
Subject Identifier	IFNγ (U/ml)					
9	1.7	2.6	24	4.9	9.4	121.2
14	6.2	60	421	88.6	254	640
19	1.3	1.6	1	5.9	10	93
21	26.6	52.1	226.4	100.8	205.2	371.5
34	10.2	22.3	163	40.7	54.7	358.4
37	31.6	31	94.2	69.5	133.1	400.1
8	340	597	1816	1024	1137	1767
39	37.6	39.7	137	69.2	141.2	494.2

IFNγ production (U/ml) by human PBMC of non-allergic subjects measured by ELISA after 4 days of culture with natural standardized cat antigen (500 BAU/ml) and exogenous recombinant IL-15 at either 0, 100 or 1000 pg/ml and exogenous recombinant IL-2 at either 0 or 10 U/ml. nd = not determined.

the presence of both cytokines. The only case where they did not synergize was subject #8 (column 6 is not higher than column 3). This subject produced the highest IFNγ response of all the subjects and may have been incapable of producing any higher levels despite the stimulation.

IL-15 Production

To put the results of cytokine induction by IL-15 in context it is crucial to know the levels of IL-15 which are present before and after activation/stimulation. The secreted protein levels of the cytokine in the presence or absence of stimulation are unknown at present. To date, only mRNA levels of IL-15 in mice have been reported (Doherty 1996) with the exception of IL-15 protein levels in human arthritic synovial fluid (McInnes 1996). mRNA is not always translated into protein, therefore mRNA levels are not necessarily indicative of protein levels. This is especially the case for IL-15 (Waldman) where very high levels of mRNA expression fail to yield detectable levels of cytokine.

We undertook to measure IL-15 protein produced by normal and allergic human PBMC (one subject of each) that were (i) unstimulated, (ii) stimulated with cat allergen 50 and 500 BAU/ml, and (iii) stimulated with lipopolysaccharide (LPS; a polyclonal activator) 0.1, 1.0 and 10.0 μg/ml. The IL-15 in supernatants was measured by bioassay with the CTLL cell line in the presence of anti-IL-2 to inhibit IL-2 induced proliferation of cells as described in the Materials and Methods section. The sensitivity of this assay for biologically active IL-15 was approximately 10 pg/ml. In each of the experiments carried out the level of IL-15 was undetectable (<10 pg/ml).

Monocytes and macrophages are thought to be the major cellular source of IL-15.

It is known that this cell type can be activated or "primed" by IFNγ. For this reason, when we stimulated human PBMC (2 new individuals) with 10 ng/ml LPS and 0.5% PHA (another polyclonal activator) independently we costimulated the PBMC with IFNγ at a range of 0.1 to 10 μg/ml. However, even with the IFNγ costimulus there was no production of IL-15 detected in the bioassay of the bulk culture supernatants. Similarly, if the PBMC were costimulated with IL-4 (10 to 1000 pg/ml) there was no detectable IL-15 protein production.

IL-15 Summary

Secreted IL-15 protein was not detected in the *in vitro* PBMC cultures stimulated with cat antigen or the polyclonal activators PHA or LPS by highly sensitive bioassay. If present in antigen-stimulated culture at 100-1000 pg/ml, concentrations 10-100 fold lower than those previously studied, IL-15 has the effect of increasing IFNγ, but not IL-5 or IL-10 production in a significant percentage of subjects. IL-15 can synergize with IL-2 or even replace it in terms of augmenting antigen stimulated IFNγ synthesis. Based on these preliminary data, one could speculate that IL-15 may be a Th1 promoting or supporting cytokine in antigen specific responses. More research will be required.

DISCUSSION

ALLERGY

The goal of this study was to investigate a possible association between antigen-driven cytokine production by fresh cells, antigen-specific antibodies present *ex vivo* and clinical sensitivity. We found that allergic subjects produce increased levels of IL-4, IL-10 and IgE and non-allergic subjects produce higher IFNγ and possibly specific IgG4. The positive correlation between antigen-driven IL-4 and antigen-specific IgE and between IgE and wheal intensity and the negative correlation between antigen-driven IFNγ and specific IgE support the hypothesis that Th2-like responses are associated with IgE and clinical sensitivity.

The subjects of this study met several criteria. Not only was the allergic condition of the allergic subjects established by skin test, RAST and a clinical history of cat allergy, but they were not on any medication for their allergy and had never undergone immunotherapy. Either medication or previous immunotherapy could have influenced the response of the PBMC or the plasma antibody levels derived from these subjects and been a confounding factor. Numerous medications are designed to reduce the induction or expression of inflammation. Immunotherapy may increase the IFNγ response and decrease the IL-4 and IL-5 response of allergen specific PBMC stimulated with polyclonal activation (Jutel 1995). Immunotherapy has also been extensively studied for effects on antibody production and many studies have demonstrated either decreased IgE or increased IgG4 after immunotherapy. Thus previous immunotherapy treatment of our subjects would have been a potential source of error when attempting to measure their natural responses to cat allergen. Other studies (Tang 1993) have intentionally recruited

allergic subjects with very high serum IgE levels (minimum 600 U/ml IgE) however this may distort the results by selective biases in recruitment. None of our subjects were found to have IgE levels of this magnitude, which reinforces how infrequent such levels are.

To objectively measure the clinical sensitivity of the allergic subjects is and remains an important goal. Instead of subjective symptom scores which are extremely difficult to quantitate, our subjects were assigned clinical sensitivity on the basis of the size of the wheal response to the skin test measured by digitized trace. As is sometimes found clinically, a number of the recruited subjects with no history of cat allergy tested positive to the skin test and were excluded from the study for this reason, as a positive skin test in the absence of clinical history is not consistent with a clinical diagnosis of immediate hypersensitivity. Not surprisingly we found considerable heterogeneity of skin test intensity in the allergic population.

The measurement of antibody isotypes directly *ex vivo* removes the difficulty of extrapolating *in vitro* data to the *in vivo* situation, in this case the clinical sensitivity. The choice of ELISA technique makes it possible to have a specific, sensitive measurement of the antibody. Many former studies relied on radiolabelled antigen-binding immunoassays using ng of antigen and found IgG in 20-30% of normals. It has been suggested that ELISAs which use μg of antigen are sensitive enough to detect low affinity or low levels of antibodies which is why recent ELISA studies can detect IgG1 in the majority of non-allergic subjects (Kemeny 1989). It was necessary to rigorously test the specificity of the IgE ELISAs as described in the Materials and Methods section, because IgE is a very low level isotype and antibodies specific for antibody Fc regions (monoclonals as well as

polyclonals) are known to exhibit some cross reactivity for other isotypes. With the specificity issue resolved the ELISA became a powerful diagnostic tool capable of detecting not only the higher levels witnessed in the allergic subjects, but for demonstrating the low levels of total and cat-specific IgE in the non-allergic subjects. The finding that non-allergic subjects produced IgE specific for cat allergen is important. Only a limited number of studies have previously identified specific IgE in clinically non-sensitive individuals. This specific IgE demonstrates that non-allergic individuals respond to allergen and that some levels of IgE are safe in the sense that they are not sufficient to lead to symptoms of allergy. Total IgE was measured as well, mostly for purposes of comparison to other studies. Both specific and total IgE were significantly elevated in allergic subjects compared to controls. The difference between allergic and non-allergic subjects was more pronounced and more statistically significant in terms of specific IgE than total IgE as would intuitively be expected, because in the conventional model of allergy only specific IgE can be cross-linked by allergen. The importance of measuring specific IgE instead of total IgE where possible must be regarded.

Specific IgG4 measured by ELISA was surprisingly bordering on significant difference with the higher levels observed in the non-allergic subjects. Most previous studies have found the opposite (Kemeny 1989; Muller 1989; Tikkainen 1989; Peng 1990; Tsai 1990; Jeanin 1994). There are many possible explanations, because there are many differences between studies. Some allergens are seasonal, others are avoidable, as opposed to cat allergen which is not seasonal and is unavoidable. However, exposure to cats can be somewhat controlled in contrast to exposure to pollens which everyone

breathes. Therefore in our study all our subjects are exposed to the allergen, however, the allergic subjects have probably attempted to limit their exposure. Other studies include allergic subjects who are occupationally (therefore more highly) exposed to the allergen. Indeed the study of novice asymptomatic bee keepers by Aalberse et al. reported that the IgG4 nature of the IgG response grew over time, presumably with exposure. Other considerations are that our allergic subjects were not selected for high IgE as has been done in some studies and that the assay techniques vary. We have used an ELISA while some others have used the radioimmunoassay. The higher level of IgG4 in non-allergic subjects would appear to support the protective theory over the anaphylactic theory for IgG4 based on this result alone, but the fact that the difference is at the border of statistical significance (despite analysis of over 80 subjects) and the contradictory data in the literature indicates that considerable caution should be exercised in attributing any role, beneficial or harmful, to IgG4 responses in normal or atopic subjects.

The heterogeneity and the overlap seen in all the isotypes is typical of human studies because of diverse genetic backgrounds. It makes diagnosis of allergy solely on the basis of antibody detection very crude and unreliable.

Much of the work on cytokine production by any type of subjects has come from T cell clones (as opposed to fresh cells), stimulated with either antigen or polyclonal stimulators or from fresh cells stimulated with polyclonal activators or from antigen specific cells (as selected by growth of the repertoire in media containing antigen) stimulated with polyclonal activators. Each approach has some inherent drawbacks. Results of studies with clones cannot be extrapolated to fresh *in vivo* cells with

confidence, and the clones were influenced by the media in which they were grown. Polyclonal activators do not stimulate through the same receptors as antigen and therefore can elicit different signals and responses. Other investigators must often choose one of these techniques because they require production of higher levels of cytokine synthesis which may be detected by relatively insensitive assays. Our study has the advantage of allowing us to measure antigen-stimulated responses by fresh cell populations in short term culture, because our cytokine assays have the specificity to detect the extremely low levels of cytokine synthesis stimulated by this more physiologic procedure.

The sensitivity of the assay for IL-4 in particular is rare. It is a bioassay instead of an ELISA which amplifies the response. Typical ELISA techniques available commercially detect approximately 30 pg/ml. This bioassay typically detects 1-2.5 pg/ml. Also it is a bioassay used in conjunction with MTS instead of tritiated thymidine uptake providing further sensitivity, because MTS detects not just dividing or proliferating bioassay detector cells, but all live cells.

The cytokine synthesis in response to cat antigen was significantly different between the allergic and non-allergic individuals. The IFNγ production was higher from non-allergic individuals while the IL-4 and IL-10 production was higher from allergic subjects. IFNγ is considered a Th1 cytokine while IL-4 is considered a Th2 cytokine. Therefore, interpreted according to the model, the results indicate that non-allergic subjects produce a more Th1-like response in comparison to the more Th2-like response of the allergic subjects. This was also found with grass pollen stimulation (Imada 1995) and with Lol p stimulation (Gagnon 1993). Our study had a considerable number of

allergic subjects (44) and has the added information of the IL-10, antibodies and clinical sensitivity. The significance of higher IL-4 production by allergics might be that IL-4 causes B cell switching to IgE as shown in *in vitro* studies or its other proinflammatory effector functions as discussed in the Th2 and Allergy section of the Introduction. The significance of higher IFNγ is that it suggests that there is no shortage of or inherent defect in the T cells specific for cat antigen peptides in the repertoire of non-allergic individuals.

The finding that IL-10 is produced at higher levels by allergen-stimulated PBMC of allergic subjects is new. The significance of this finding lies in the fact that both Th1 and Th2 human clones have been shown to produce IL-10 and both Th1 and Th2 clones were suppressed by IL-10 (Del Prete 1993). The higher IL-10 production by the Th2-like response of the allergic subjects could be interpreted as evidence for IL-10 as a Th2 cytokine in the human as it is in the murine system. It makes interesting speculation to say that higher IL-10 production by allergic subjects could suppress the IFNγ response by these subjects. However why would the IL-4 response not similarly be suppressed if IL-10 suppresses both Th1 and Th2 fresh cells as it does clones? It could also be interpreted as a difference in the APC of the subject groups since IL-10 is also produced by B cells and macrophages which are part of the PBMC composition along with T cells. APC type and B7 expression affect IL-4 production by $CD4^+$ T cells. Secrist et al. (1995) found that B cells are better than monocytes at inducing IL-4 production, while monocytes induce more IFNγ than B cells. B7 expression by APC preferentially enhanced IL-4 and IL-5 and decreased IFNγ production by primed cells (Yang 1995). If differences in IL-10

production reflect differences in APC type then it may be of significance.

The antigen-stimulated cytokine production is sharply contrasted by the cytokine production in response to PHA. PHA elicited higher IL-10 from non-allergic PBMC than allergic PBMC and IFNγ levels which were not significantly different between the two groups. This demonstrates that polyclonal activation is not a credible substitute for antigen, a finding that is consistent with the findings of Imada et al. (1995). It also demonstrates that allergic individuals do not respond to everything in a Th2-dominant manner or have a qualitative defect in their capacity to produce IFNγ. The IFNγ response of either group was definitely stronger than that in response to antigen, however the IL-10 response was weaker. Given that PHA theoretically stimulates all T cells, as opposed to less than 1% of T cells that are stimulated by cat antigen(s), the higher PHA driven IFNγ response is expected. It also weakly suggests that the cat antigen itself was not a polyclonal activator because if it were the response would have been much stronger. The fact that IL-10 production by allergic subjects was lower in response to PHA than to cat antigen suggests that the source of the IL-10 in response to cat antigen was not T cells but rather the APC already mentioned, which would not be similarly stimulated by PHA because it only affects T cells.

BLOCKING ANTIGEN PRESENTATION

Chloroquine treatment of the PBMC in culture was done to test the antigen specificity of the response to antigen. Chloroquine, through interference with lysosomal enzymes and vesicle fusion, disrupts the process of antigen processing and presentation to T cells. It did indeed reduce the IFNγ and IL-10 responses to cat antigen consistent with

the cat antigen preparation being antigenic as opposed to mitogenic. Interestingly it completely blocked IFNγ but only partially inhibited IL-10 possibly indicating again that the source of IL-10 is not solely T cells in this system. It also very substantially reduced the IFNγ and IL-10 responses to PHA. Presumably this result was due to decreased IL-2 production by T cells and decreased responsiveness of T cells to IL-2 as described by Landewé (1995).

Other possible treatments include OKT4 which binds CD4 on the T cell and would block antigen presentation (not processing). This alternative was used by Imada et al. 1995. Our alternative in this study was to add an anti MHC Class II antibody cocktail to culture which should also block antigen presentation. In practice it did block IFNγ but not IL-10. In fact, it appears that IL-10 levels increased in cultures where the cocktail was employed. Antibodies of irrelevant specificities were applied as controls and did not consistently block IFNγ to the same extent. This indicated that the cat antigen was eliciting antigen-specific responses and not causing non-specific activation. However the fact that the IL-10 response was not blocked indicates that the IL-10 source is not T cells, and that the antibody may have stimulated the APC to which it binds to produce IL-10. This hypothesis would require further testing.

An interesting finding was that an antibody to the MHC Class I was also able to block the IFNγ response. This result was confirmed after rigorous testing to ensure that there was no non-specific toxicity associated with this reagent. The suggestion that the MHC Class I pathway is involved in the response to cat antigen requires confirmation. Another approach in future may be to deplete $CD8^{+}$T cells from the PBMC or add

antibodies against CD8 to culture and assess the effect on cat antigen stimulated cytokine production, in particular IFNγ. It would also be interesting to try to isolate cat antigen specific $CD8^{+}$T cells. In future it would also be important to confirm use of the MHC Class I pathway with other exogenous test antigens.

Other work has previously suggested a role for MHC Class I in exogenous antigen presentation. Harding and Song (1994) found that particulate OVA injected intravenously (coupled to latex particles) was presented by murine macrophage MHC Class I ($H\text{-}2K^{b}$) whereas soluble OVA was not. Norbury et al. demonstrated MHC Class I presentation of soluble OVA by murine macrophages (1995), and Malaviya et al. demonstrated that murine mast cells, which are not known to present MHC Class II, could phagocytose and kill live bacteria, subsequently presenting a fusion protein expressed within the bacteria via MHC Class I and elicit a T cell hybridoma response (1996). Notably the previous findings have been confined to the murine system. In our system (human PBMC) the possible cellular sources of MHC Class I presentation are B cells, T cells and monocytes. Monocytes are the more likely suspect since the previous work in this field has found that macrophages and cells with phagocytic capacity are capable of presenting exogenous antigen in MHC Class I.

Many questions remain regarding a mechanism for MHC Class I presentation of exogenous peptide. Possibilities include direct delivery of exogenous antigen to the cytosol, or intracellular compartment release of antigen or peptide into the cytosol, or lysis of the intracellular compartment, or MHC Class I entry into the intracellular compartments, or regurgitation of peptides released from MHC Class II molecules on the

cell surface becoming bound to empty MHC Class I molecules still on the cell surface. However substantiation of these possibilities is lacking.

CORRELATIONS BETWEEN CYTOKINE PRODUCTION, ANTIBODY SYNTHESIS AND CLINICAL SENSITIVITY

The antigen-specific antibody and antigen-driven cytokine parameters were tested for non-parametric correlations in order to test the hypothesis that Th2-like responses to allergen are associated with allergen-specific IgE and clinical sensitivity. Correlations between antibody isotypes as well as between different cytokines were also tested to explore possible regulatory information.

It was established that specific IgE concentration correlated to clinical sensitivity as measured by the intensity of the wheal reaction, however total IgE did not correlate to the wheal data. This is an important result which must be considered when surveying work conducted by others who measure total IgE as a diagnostic indication of allergy status or improvement after some form of treatment, such as immunotherapy. The overlap of total IgE between allergic and non-allergic individuals further emphasizes this point. It must also be considered however, that overlap of cat-specific IgE levels between subject groups was noted as well, and that the correlation (which does not prove cause and effect) between cat-specific IgE and the wheal, though significant (p= 0.004), was not as strong an association as required to diagnose allergy or its severity on the basis of cat-specific IgE either. Indeed, multiple studies have indicated that clinical results are not

always improved when specific IgE levels decrease.

The specific IgG4 levels did not correlate with the wheal either. If the dominant theory that IgG4 is a 'blocking antibody' were true one might expect to see a negative correlation in these parameters. Similarly if IgG4 were an anaphylactic antibody one might expect a positive correlation between these parameters. However these correlations are tested on outbred human subjects which supply innumerable genetic and environmental variables, and testing the IgG4 of the population as a whole against the wheal reaction of the population as a whole would be influenced by these factors. If results were obtained from individuals over time courses there would be an opportunity to observe whether increases or decreases in particular antibody isotypes were associated with changes in clinical sensitivity. Placebo subjects in an immunotherapy study might provide useful data for this topic. Without a correlation between the IgG4 and the wheal it would be presumptuous to conclude from the borderline significant finding that IgG4 is higher in non-allergic subjects that it is a protective blocking antibody.

An interesting correlation we observed was that of specific IgE and specific IgG4 in the non-allergic subjects. What made this finding more intriguing was that it was not significant in the allergic population. B cells switch to IgE and IgG4 under the influence of the same cytokines. IL-4 induces the switch to these isotypes while IFNγ opposes it. With the only known regulatory influences conceived to be the same for both isotypes it is not surprising to find the isotypes correlated. That the correlation was not found in the allergic population was somewhat surprising, but it was also found in an earlier study reported by Peng et al. (1992). This study further reported that a correlation between

ragweed specific IgG4 and specific IgE developed in the same subjects after two years of immunotherapy. Without non-allergic subjects available for comparison the significance of this finding was not fully realized. Our demonstration that there is a correlation in non-allergic individuals suggests that the immunotherapy influence was positive. In our study the allergic subjects mostly fall in the category of having a lower ratio of IgG4:IgE than the non-allergic subjects, but other studies have reported allergic subjects who have elevated IgG4 but not elevated IgE . Only 9 of 23 bakers with complaints of allergy to wheat flour had specific IgE but all had detectable specific IgG4 and that IgG4 was higher than that seen in control subjects (Tikkainen 1989). Possibly this is an indication that the balance of the isotypes rather than absolute quantity of either is the relevant factor. In fact in a study by James et al. the 11 children who lost their allergy to cow's milk had a lower ratio of IgE:IgG4 before and after clinical improvement than the 18 children who retained their allergy to cow's milk (1992). The difference between our two subject groups raises the questions of whether the balance of these two isotypes is important in determining allergy or whether the loss of that balance is a symptom of allergy. More investigation is required before these questions can be answered, however in the interim it is possible to speculate.

Mast cells bind IgE with high affinity via the Fc_cR and IgG4 with low affinity via the $Fc_\gamma R$ which may allow for cross linking of allergen by either or both isotypes simultaneously. A recent study by Kobayashi et al. (1996) found differences in the specificity of polyclonal IgG4 and polyclonal IgE from the same source by testing the antibody preparations for binding to overlapping peptides of the specific allergen. It is

conceivable that particular balances of the antibodies lead to a particular situation on the mast cell surface which influences the optimal crosslinking required to signal mast cell degranulation.

The correlations tested between cytokine and antibody revealed interesting findings as well. A correlation between IL-4 and specific IgE might be expected due to *in vitro* studies where addition of exogenous IL-4 in culture resulted in increased total IgE production while addition of anti IL-4 resulted in decreased IgE as compared with that of controls. These studies were instrumental in determining that IL-4 is a switch factor for IgE. Since that discovery it has been thought that aberrantly high IL-4 production in allergic individuals was responsible for the high specific IgE and therefore clinical sensitivity. However, measurement of antigen-induced cytokine production by fresh cells is very rarely carried out (due to the technicalfactors discussed above) and has not been previously tested for a correlation to specific *ex vivo* IgE. Similarly a negative correlation between IFNγ and IgE may have been expected as a result of studies where it was reported that IFNγ opposes IL-4 in inducing the switch to IgE. Our data indicate positive correlations between antigen-driven IL-4 and total IgE and between antigen-driven IL-4 and specific IgE in the population as a whole. They also indicate a negative correlation between IFNγ and specific IgE in the whole population, and therefore to the ratio of IFNγ:IL-4 (our best measure of Th1 vs. Th2). Possibly allergen-driven cytokines will serve as objective parameters by which allergy can be diagnosed in future.

Some other investigators have looked for a correlation between IL-4 and IgE in allergic and non-allergic individuals. Van der Pouw Kraan et al. reported a correlation of

polyclonally activated IL-4 and total IgE induced in vitro in non-allergic individuals, but not in allergic individuals. Our methods differ from theirs in the cytokine stimulation and in that we measure specific IgE ex vivo without stimulus. They did not report results of correlation tests of the whole population as we have done.

In our study IL-4 and IgE in the allergic selection of the population alone did not correlate. Neither did IFNγ and cat-specific IgE. This was surprising. It indicates that in the allergic population allergen-driven *in vitro* IL-4 or IFNγ and specific *ex vivo* IgE are not directly related. This could have implications for assessing treatment progress of allergic individuals and suggests that cytokines may not be appropriate parameters to measure the severity of allergy.

Further inspection of this phenomenon reveals that the allergic subjects with the lowest IL-4 never have high specific IgE and the subjects with the highest IFNγ never have high specific IgE. The cytokine data is derived from identical stimulation of all subjects under identical *in vitro* conditions, however the specific IgE is measured *ex vivo* and is influenced by the different environmental conditions of the subjects. For further pursuit of this study it might be interesting to measure *in vitro* IgE after antigen stimulation and test this measurement of IgE for a correlation to the *in vitro* antigen-driven IL-4.

It has been suggested that IL-13 is also very important in switching B cells to produce IgE, and could be another important factor in allergy. Studies have shown that IL-13 is not as potent in switching B cells as IL-4 (Punnonen 1993, 1994). However relative concentrations of the two cytokines could reflect more importance for IL-13 since

antigen-driven IL-4 is usually produced in low concentrations which are virtually undetectable in non-allergic subjects, and depending on the assay sensitivity in allergic subjects as well. It is possible that IL-13 and IL-4 together would correlate to IgE, but that would require further study.

Despite evidence that IL-4 induces the B cell switch to IgG4 and IFNγ inhibits that switch (Sutherland 1993) we did not observe a correlation between either cytokine and specific IgG4. What other signals or factors may be regulating IgG4 are unknown.

IFNγ and IL-4 were not correlated. This may argue that they have little effect on mature cells as opposed to the drastic effects each has on naive T cell differentiation to Th1 or Th2.

Due to studies with human clones which showed that IL-10 suppressed both Th1 and Th2 (Del Prete 1993) it might be expected that IL-10 negatively correlate to the other cytokines, but IL-10 was not correlated to IFNγ at all and was positively correlated to IL-4. This might be interpreted as evidence suggesting that IL-10 is a Th2-like cytokine (like IL-4) and not a Th1-like cytokine (like IFNγ) and that at these concentrations of IL-10 it is not a suppressive cytokine. Researchers often employ high concentrations of exogenous cytokine in culture and assume or speculate that similar functions occur with lower concentrations of the endogenous cytokine. The validity of this assumption remains to be tested.

The APC source of IL-10 must also be considered, because several lines of evidence indicated that T cells were not the only source of IL-10 in this system, and the type of APC does affect the T cell response (Secrist 1995). Do high IL-10 producing

APC preferentially activate or promote differentiation into IL-4 producing Th2 effector cells? The difference between clones and fresh cells must also be considered, because the observation of suppression of clones does not necessarily mean that fresh cells will also be suppressed. Therefore the significance of the correlation between IL-4 and IL-10 is still unclear.

In summary this study has shown that non-allergic individuals do respond to cat antigen with cytokine production and produce antibodies specific for cat antigen, however they do not display clinical sensitivity. The allergic subjects display positive skin tests, elevated IgE (total and specific), possibly lower cat specific IgG4, and produce a more Th2-like response to cat antigen challenge *in vitro* (higher IL-4 and lower IFNγ). The antigen specificity of the response was confirmed by tests with chloroquine and anti MHC Class II antibodies. Interestingly anti MHC Class I antibodies also blocked antigen specific cytokine responses. The population as a whole displays a positive correlation between antigen-driven IL-4 and specific IgE and a negative correlation between IFNγ and specific IgE, arguing that Th2-like responses are associated with IgE production. The specific IgE correlated with the wheal intensity and therefore we conclude that antigen-driven cytokine production, antigen-specific IgE and clinical sensitivity are directly related.

IL-15

IL-15 was discovered with T cell stimulatory activity (Grabstein 1994). It was found to support the growth of both $CD4^+$ (Th0, Th1, and Th2) and $CD8^+$ antigen-dependent T cell clones, as well as activated normal T cells and gamma delta T cells. Results obtained by use of clones must be regarded with some caution, and even the 'normal' T cells were not activated physiologically, but with PHA. It remains an important goal to assess the effects of IL-15 on fresh antigen-activated cells.

There are different approaches available for measuring responses. Proliferation responses are indicative of the intensity of the stimulus, whereas cytokine production is more indicative of the character of the response. The cytokine production by T cells or even clones stimulated with IL-15 has not been examined, except in the study by Seder et al where the cytokine assays were not sensitive enough to detect antigen-driven cytokine synthesis in the majority of their subjects. In particular, we were interested in whether IL-15 would enhance a particular T helper response ie. Th1 or Th2. The role of IL-2 in Th1 responses as opposed to Th2 responses has been characterized recently in the murine system as required for IFNγ but not IL-4 synthesis from Ag-primed T cells (Yang 1993), and preliminarily in the human systems as an antigen-specific Th1 augmenting but not required cytokine with no effect on Th2 (Imada in preparation). The IL-15R shares signalling components of the IL-2R leading to our speculation that IL-15 may, similar to IL-2, augment human Th1-dominated antigen-specific responses.

In testing the ability of IL-15 to augment human Th1 or Th2 antigen-specific responses we cultured fresh PBMC with or without antigen in the presence or absence of

exogenous rIL-15 and measured the synthesis of several cytokines. The subjects were non-allergic to the cat antigen used as the stimulus, and in the previous study this group was determined to have virtually undetectable IL-4 but substantial (typically 10 to 50 U/ml) IFNγ and IL-10 synthesis when stimulated with this antigen. The unstimulated cultures did not produce higher levels of IFNγ or IL-10 when cultured with rIL-15. In contrast, the cultures stimulated with cat antigen produced higher levels of IFNγ if IL-15 was present in culture. The levels of antigen-driven IL-10 were not substantially different in the presence of IL-15. Nor was there a detectable influence on antigen-driven IL-5 production.

The results indicate that IL-15 (used at the concentrations tested) does not cause non-specific production of IFNγ or IL-10 from fresh cells, but does increase IFNγ production in response to antigen exposure. Previously in the literature there was no effect of exogenous IL-15 on non-specific or antigen-specific cytokine responses of PBMC reported. Seder et al. were measuring antigen driven cytokine synthesis by PBMC in the presence or absence of IL-15, but found no significant change. Their system and ours differed in antigens; their system stimulated with tetanus toxoid, whereas our model stimulates with cat antigen, but both tetanus toxoid and cat antigen are considered recall antigens. Additionally we differed in the concentrations of IL-15 employed (1-10 ng/ml in the Seder study versus 10-1000 pg/ml in ours). Most importantly, PBMC produced detectable IL-10 and IFNγ cytokine in response to cat antigen in our system, whereas the tetanus toxoid antigen did not elicit detectable IL-2, IL-4 or, in the majority of subjects, detectable IFNγ in their system. The sensitivities of the assays may have been very

important in this discrepancy, and could have prevented recognition of changes in cytokine concentrations due to IL-15 in their system.

The significance of our results is that we demonstrate a shift in antigen-driven cytokine response by PBMC due to IL-15. The prominent role of IFNγ in the Th1 response and the increase in the production of this cytokine attributed to IL-15 combined with undetectable effects on IL-5 or IL-10 suggests that IL-15 supports or promotes a Th1-like response. If it is confirmed that IL-15 promotes Th1-like responses this will be yet another *in vitro* function in common with IL-2, however an important difference is that high levels of IL-2 led to non-specific activation of cytokine synthesis (Lefkovits 1984). In contrast to T cells which we have shown are affected by IL-15 in antigen-specific responses only, non-specific polyclonal B cell Ig secretion is costimulated by CD40L and IL-15 (Armitage 1995). The power to direct T helper responses to antigen is desired for many disorders where the Th1 / Th2 balance is considered inappropriate. IL-15 may prove to be an attractive alternative to IL-2.

The non-specific activation of cytokine production by high levels of IL-2 compared to no non-specific cytokine production induced by IL-15 may have implications for current work on gene therapy for cancer with the IL-2 gene.

Whether IL-15 is capable of augmenting Th1 cytokine production from a Th2-like response is unanswered. Very preliminary work investigating the influence of IL-15 on the Th2-like response of cat allergic subjects to cat antigen has begun and was included in the preceding data tables, but is not sufficient for speculation. In addition to measuring the possible increase in IFNγ production it will be important to measure IL-4 (a prominent

Th2-like cytokine) for possible changes also. There is an ongoing debate and investigation into whether polarized T helper profiles can spontaneously, or through some manipulation, be shifted toward the opposite extreme. It appears IL-15 will become part of this debate.

The possibilities that either IL-2 or IL-15 are dependent on the other, or that they antagonize each other, or that they synergize in action, as may be expected due to the shared receptor components, have not been fully addressed. Some data have been accumulated on the interrelated roles of IL-2 and IL-15 in their various functions. NK cell proliferation is induced by either IL-2 or IL-15 and the two cytokines were shown to synergize in this function until the receptor was saturated (Carson 1994). IL-2 and IL-15 synergized to induce $CD4^+$ TDAC proliferation, but not with respect to cytokine synthesis (Lewko 1995). A decrease in IFNγ and GM-CSF occurred if both IL-15 and IL-2 were present in culture despite the fact that either IL-2 or IL-15 alone in culture increased the production of IFNγ and GM-CSF. Supporting or promoting Th1 responses is a newly discovered function of IL-15 and therefore has not been tested.

In our studies we assessed whether IL-15 required the presence of IL-2 by comparing the effects of IL-15 in the presence and absence of an anti IL-2 monoclonal antibody (Cetus). We also examined whether IL-15 and IL-2 synergize in augmenting the IFNγ response to antigen by adding both rIL-15 and rIL-2 to antigen-stimulated cultures and comparing these results to those obtained with either cytokine alone. The results indicated that IL-15 continued to augment IFNγ while endogenous IL-2 was neutralized. Sometimes the IFNγ remained optimal, but results from other subjects were suboptimal indicating that endogenous IL-2 had an effect in those cultures. The synergism

experiments revealed that IL-2 and IL-15 do indeed synergize in augmenting IFNγ synthesis by PBMC stimulated with cat antigen. Thus, IL-15 does not require IL-2, but is capable of synergizing with it to induce higher IFNγ synthesis.

These studies did not test for a requirement on the part of IL-2 for endogenous IL-15. To do so an antibody against IL-15 could be utilized in the same manner as the antibody against IL-2 was to determine whether IL-15 required IL-2. Given the low, uncommon production of IL-15 as we understand it and the common and widespread effects of IL-2, this would seem an unlikely requirement.

The observation of synergy *in vitro* does not suggest that there is necessarily synergy *in vivo*. The different cellular sources of the two cytokines, and the current lack of understanding regarding which conditions lead to production of active IL-15 protein make it very difficult to predict or even speculate whether these two cytokines are present together *in vivo* with the opportunity to synergize.

Indeed all functional studies with IL-15 are difficult to interpret, because so little is known of where, when and why biologically active IL-15 is produced. Many studies have focussed upon production of IL-15 mRNA, but only one study has reported IL-15 protein (McInnes 1996). The mRNA studies have indicated that the adherent population of PBMC (monocytes) is a good source of IL-15 (Grabstein 1994). We undertook to measure IL-15 synthesis by PBMC under various stimulatory conditions by applying the culture supernatants to a bioassay sensitive to 10 pg/ml IL-15. The stimuli were: (i) no stimulus; (ii) cat antigen; and (iii) LPS (a polyclonal activator). None of these stimuli resulted in detectable synthesis of IL-15 at any of a range of harvest times. Further

attempts with LPS and PHA (another polyclonal activator) as independent stimuli in the presence or absence of IFNγ or IL-4 costimuli did not result in detectable IL-15 either.

We add to the literature that human PBMC do not produce greater than 10 pg/ml IL-15 protein in response to these polyclonal activators or recall antigen. It will be important to the overall understanding of the roles of IL-15 to continue to test other sources for synthesis of IL-15 protein. It would be particularly interesting to measure IL-15 protein in the bone marrow and thymus since it was found that bone marrow and thymic stromal cell lines produced IL-15R mRNA but not IL-2R mRNA (Giri 1995).

There are currently many functions of IL-15 identified *in vitro,* but very little evidence exists to suggest what the role of IL-15 is *in vivo*, because aside from the usual difficulties of extrapolating *in vitro* data to the *in vivo* situation, IL-15 protein has not been detected, with the exception of rheumatoid arthritis patient synovial fluid (McInnes 1996). Studies with anti IL-15 or anti IL-15R will be an important addition. It is possible that endogenous IL-15 may be present and active yet undetectable by any current assays, or so unstable, or of such high affinity that it is quickly removed and only detectable for a very short time. Despite any of these possibilities, if active IL-15 is present, then neutralizing it with antibodies will produce a noticeable effect. This may be the best method for determining which of the *in vitro* roles and functions of IL-15 actually have relevance *in vivo*. Some of the difficulties of this type of approach are that the antibodies against the cytokine itself may be cross reactive and antibodies to the receptor may block other cytokines which bind to the same receptor. There are only limited reports to date of use of anti IL-15 antibodies. One report from McInnes et al (1996) noted a decrease in T

cell polarization *in vitro* due to the neutralization of IL-15 and IL-8 simultaneously, which was not neutralized by either antibody alone. Another report from Nishimura et al (1996) noted that anti IL-15 abrogated the proliferation and cytokine synthesis (IFNγ and IL-4) by γδ T cells infected with *Salmonella choleraesuis.* These types of studies may be the best approach to identifying biologically active and relevant IL-15.

Once active IL-15 is found and characterized it would be possible to add potential inhibitors and enhancers to the experiments and investigate the regulatory influences on this cytokine. IL-10, known to potently inhibit IL-2, was shown to increase IL-15 mRNA, suggesting that IL-15 may not be as easily downregulated as IL-2 (Doherty 1996). Neither were other macrophage inhibitory cytokines effective at reducing IL-15 mRNA. It has been suggested that IL-15 is stored at high levels within cells in its mRNA form ready for rapid release in response to environmental or pathogenic stimuli (Tagaya 1996). It could be further speculated that IL-15 is an important induction cytokine of natural immunity produced by macrophages and capable of activating NK and γδ T cells, and chemoattracting T cells for the adaptive immune response, yet also long lasting and capable of supporting T cell proliferation after IL-2 activity has been reduced. If so, production of IL-15 may only be detectable after pathogenic stimuli.

In summary, IL-15 is a tightly regulated cytokine produced by monocyte/macrophage, epithelial and fibroblast cell types under unknown conditions with the abilities identified *in vitro* to activate proliferation and either cytokine or immunoglobulin secretion from NK cells, γδ and αβ T cells, or B cells through the IL-15R which includes components of the IL-2R. The shared receptor components appear to

explain the similarities in function found between IL-2 and IL-15, however, discussion regarding another receptor for IL-15 has been raised (Tagaya 1996). We have demonstrated that IL-15 augments established Th1 antigen-dependent responses without increasing non-specific cytokine production, and that it does not require, but is able to synergize with IL-2 in this capacity. The differences between IL-2 and IL-15 include cellular source, receptor distribution, expression, and regulation likely leading to different *in vivo* functional roles for these seemingly similar cytokines.

REFERENCES

Aalberse R. C., van der Gaag R., van Leeuwen J. 1983. Serological aspects of IgG4 antibodies. I. Prolonged immunization results in an IgG4-restricted response. *J. Immunol.* 130:722

Anderson D. M., Johnson L., Glaccum M., Copeland N. G., Gilbert D. J., Jenkins N. A., Valentine V., Kirstein M. N., Shapiro D. N., Morriss S. W., Grabstein K., Cosman D. 1995. Chromosomal assignment and genomic structure of IL-15. *Genomics* 25:701

Armitage R., Macduff B. M., Eisenman J., Paxton R., and Grabstein K. H. 1995. IL-15 has stimulatory activity for the induction of B cell proliferation and differentiation. *J. Immunol.* 154:483

Carson, W. E., Giri, J. G., Lindemann, M. J., Linett, M. L., Ahdieh, M., Paxton, R., Anderson, D., Eisenman, J., Grabstein, K., Caligiuri, M. A. 1994. Interleukin (IL) 15 is a novel cytokine that activates human natural killer cells via components of the IL-2 receptor. *J. Exp. Med.* 180:1395

Chaturvedi P., Yu Q., Southwood S., Sette A., Singh B. 1996. Peptide analogs with different affinities for MHC alter the cytokine profile of T helper cells. *Int. Immunol.* 8:745

Chretien I., Pene J., Briere F., de Waal Malefijt R., Rousset F., deVries J. E. 1990. Regulation of human IgE synthesis. I. Human IgE synthesis *in vitro* is determined by the reciprocal antagonistic effects of interleukin 4 and interferon-γ. *Eur. J. Immunol.* 20:243

Coyle A. J., Wagner K., Bertrand C., Tsuyuki S., Bews J., Heusser C. 1996. Central role of Immunoglobulin (Ig) E in the induction of lung eosinophil infiltration and T helper 2 cell cytokine production: Inhibition by a non-anaphylactogenic anti-IgE antibody. *J. Exp. Med.* 183:1303

Del Prete G. F., De Carli M., Almerigogna F., Giudizi M-G., Biagiotti R., Romagnani S. 1993. Human IL-10 is produced by both type 1 helper (Th1) and type 2 helper (Th2) T cell clones and inhibits cytokine production. *J. Immunol.* 150:1

Delespesse G., Sarfati M., Hofstetter H. 1989. Human IgE-binding factors. *Immunol. Today* 10:159

Doherty T. M., Seder R. A., and Sher A. 1996. Induction and regulation of IL-15 expression in murine macrophages. *J. Immunol.* 156:735

Esnault S., Benbernou N., Lavaud H., Shin H. C., Potron G., Guenounou M. 1996. Differential spontaneous expression of mRNA for IL-4, IL-10, IL-13, IL-2 and interferon-

gamma (IFNγ) in peripheral blood mononuclear cells (PBMC) from atopic patients. *Clin. Exp. Immunol.* 103:111

Fagan D. L., Slaughter C. A., Capra J. D., Sullivan T. J. 1982. Monoclonal antibodies to immunoglobulin G4 induce histamine release from human basophils in vitro. *J. Allergy Clin. Immunol.* 70:399

Gagnon R., Akoum A., Hébert J. 1993. Lol p 1-induced IL-4 and IFNγ production by peripheral blood mononuclear cells of atopic and nonatopic subjects during and out of the pollen season. *J. Allergy Clin. Immunol.* 91:950

Gergen P. J., Turkeltaub P. C., Kovar M. G. 1987. The prevalence of allergic skin test reactivity to eight common aeroallergens in the US population: results from the second National Health and Nutrition Examination Survey. *J. Allergy Clin. Immunol.* 80:669

Giri J. G., Kumaki S., Ahdieh M., Friend D. J., Loomis A., Shanebeck K., DuBose R., Cosman D., Park L. S., and Anderson D. M. 1995. Identificatio and cloning of a novel IL-15 binding protein that is structurally related to the α chain of the IL-2 receptor. *EMBO J.* 14:3654

Giri J. G., Anderson D. M., Kumaki S., Park L. S., Grabstein K. H., and Cosman D. 1995. IL-15, a novel T cell growth factor that shares activities and receptor components with IL-2. *J. Leuk. Biol.* 57:763

Giri J. G., Ahdieh M., Eisenman J., Shanebeck K., Grabstein K. H., Kumaki S., Namen A., ParkL. S., Cosman D. and Anderson D. 1994. Utilization of the β and γ chains of the IL-2 receptor by the novel cytokine IL-15. *EMBO J.* 13:2822

Gonzalez-Noriega A., Grubb J. H., Talkad V., Sly W. S. 1980. Chloroquine inhibits lysosomal enzyme pinocytosis and enhances lysosomal enzyme secretion by impairing receptor recycling. *J. Cell Biol.* 85:839

Grabstein K. H., Eisenman J., Shanebeck K., Rauch C., Srinivasan S., Fung V., Beers C., Richardson J., Schoenburn M. A., Ahdieh M., Johnson L., Alderson M. R., Watson J. D., Anderson D. M., Giri J. G. 1994. Cloning of a T cell growth factor that interacts with the β chain of the interleukin-2 receptor. *Science* 264:965

Harding C. V., Song R. 1994. Phagocytic processing of exogenous particulate antigens by macrophages for presentation by Class I MHC molecules. *J. Immunol.* 153:4925

Hsieh C. S., Heimberger A. B., Gold J. S., O'Garra A., Murphy K. M. 1992. Differential regulation of T helper phenotype development by interleukins 4 and 10 in an alpha beta T cell-receptor transgenic system. *Proc. Natl. Acad. Sci. USA* 89:6065

Hsieh C. S., Macatonia S. E., Tripp C. S., Wolf S. F., O'Garra A., Murphy K. M. 1993. Development of TH1 $CD4^{+}$T cells through IL-12 produced by *Listeria*-induced macrophages. *Science* 260:547

Imada M., Simons F. E. R., Jay F. T., HayGlass K. T. 1995. Antigen mediated and polyclonal stimulation of human cytokine production elicit qualitatively different patterns of cytokine gene expression. *Int. Immunol.* 7(2):229

Imada M., Simons F. E. R., Jay F. T., HayGlass K. T. 1995. Allergen-stimulated interleukin-4 and interferon-γ production in primary culture: responses of subjects with allergic rhinitis and normal controls. *Immunology* 85:373

James J. M., Sampson H. A. 1992. Immunologic changes associated with the development of tolerance in children with cow milk allergy. *J. Pediatr.* 121:371

Jeanin, P., Delneste, Y., Tillie-Leblond, I., Wallaert, B., Carlier, A., Pestel, J., Tonnel, A.B. 1994. Abnormal IgG4 antibody response to aeroallergens in allergic patients. *Int. Arch. Allergy Immunol.* 104:191

Jutel M., Pichler W.J., Skrbic D., Urwyler A., Dahinden C., Müller U.R. 1995. Bee venom immunotherapy results in decrease of IL-4 and IL-5 and increase of IFNγ secretion in specific allergen-stimulated T cell cultures. *J. Immunol.* 154:4187

Kelso, A. 1995. Th1 and Th2 subsets: paradigms lost? *Immunol. Today* 16:374

Kemeny, D. M., Urbanek, R., Ewan, P., McHugh, S., Richards, D., Patel, S., Lessof, M. H. 1989. The subclass of IgG antibody in allergic disease: II. The IgG subclass of antibodies produced following natural exposure to dust mite and grass pollen in atopic and non-atopic individuals. *Clin. Exp. Allergy* 19:545

Kersh G. J., Allen P. M. 1996. Essential flexibility in the T-cell recognition of antigen. *Nature* 380:495

Klucka C. V., Ownby D. R., Green J., Zoratti E. 1995. Cat sheddings of Fel d 1 is not reduced by washings, Allerpet-C spray, or acepromazine. *J. Allergy Clin. Immunol.* 95:1164

Kobayashi, I., Sakiyama, Y., Tame, A., Kobayashi, K., Matsumoto, S. 1996. IgE and IgG_4 antibodies from patients with mite allergy recognize different epitopes of *Dermatophagoides pteronyssinus* group II antigen (Der p 2). *J. Allergy Clin. Immunol.* 97:638

Kumaki S., Ochs H. D., Timour M., Schooley K., Ahdieh M., Hill H., Sugamura K.,

Anderson D., Zhu Q., Cosman D., and Giri J. G. 1995. Characterization of B-cell lines established from two X-linked severe combined immunodeficiency patients: interleukin-15 binds to the B cells but is not internalized efficiently. *Blood* 86:1428

Landewé R. B. M., Miltenburg A. M. M., Verdonk M. J. A., Verweij C. L., Breedveld F. C., Daha M. R., Dijkmans B. A. C. 1995. Chloroquine inhibits T cell proliferation by interfering with IL-2 production and responsiveness. *Clin. Exp. Immunol.* 102:144

Lefkovits I., Waldmann H. 1984. Limiting dilution analysis of the cells of the immune system. I. The clonal basis of the immune response. *Immunol. Today* 5:265

Lewko W. M., Smith T. L., Bowman D. J., Good R. W., and Oldham R. K. 1995. Interleukin-15 and the growth of tumor derived activated T-cells. *Cancer Biotherapy* 10:13

MacDonald S., Rafnar T., Langdon J., Lichtenstein L. M. 1995. Molecular identification of an IgE-dependent histamine releasing factor. *Science* 269:688

McInnes I. B., Al-Mughales J., Field M., Leung B. P., Huang F. P., Dixon R., Sturrock R. D., Wilkinson P. C. & Liew F. Y. 1996. The role of interleukin-15 in T-cell migration and activation in rheumatoid arthritis. *Nature Medicine* 2:175

Maggi E., Parronchi P., Manetti R., Simonelli C., Piccinni M.-P., Santoni Rugiu F., de Carli M., Ricci M., Romagnani S. 1992. Reciprocal regulatory effects of IFNγ and IL-4 on the in vitro development of human Th1 and Th2 clones. *J. Immunol.* 148:2142

Malaviya R., Twesten N. J., Ross E. A., Abraham S. N., Pfeifer J. D. 1996. Mast cells process bacterial antigens through a phagocytic route for Class I MHC presentation to T cells. *J. Immunol.* 156:1490

Marsh D. G., Neely J. D., Breazeale D. R., Ghosh B., Freidoff L. R., Ehrlich-Kautzky E., Schou C., Krishnaswamy G., Beatty T. H. 1994. Linkage analysis of IL-4 and other chromosome 5q31.1 markers and total serum immunoglobulin E concentrations. *Science* 264:1152

Matsuzawa Y., Hostetler K. Y. 1980. Inhibition of lysosomal phospholipase A and phospholipase C by chloroquine 4,4'-bis(diethyl-aminoethoxy)α,β-diethyldiphenylethane. *J. Biol. Chem.* 255:5190

Mosmann T. R., Cherwinski H., Bond M. W., Giedlin M. A., Coffman R. L. 1986. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J. Immunol.* 136:2348

Müller, U., Helbling, A., Bischof, M. 1989. Predictive value of venom-specific IgE, IgG and IgG subclass antibodies in patients on immunotherapy with honey bee venom. *Allergy* 44:412

Nishimura H., Hiromatsu K., Kobayashi N., Grabstein K. H., Paxton R., Sugamura K., Bluestone J. A., and Yoshikai Y. 1996. IL-15 is a novel growth factor for murine γδ T cells induced by *Salmonella* infection. *J. Immunol.* 156:663

Norbury C. C., Hewlett L. J., Prescott A. R., Shastri N., Watts C. 1995. Class I MHC presentation of exogenous soluble antigen via macropinocytosis in bone marrow macrophages. *Immunity* 3:783

Norman P. S. 1993 Therapeutic potential of peptides in allergic disease. *Annals of Allergy* 71:330

Openshaw P., Murphy E., Hosken N. A., Maino V., Davis K., Murphy K., O'Garra A. 1995. Heterogeneity of intracellular cytokine synthesis at the single-cell level in polarized T helper 1 and T helper 2 populations. *J. Exp. Med.* 182:1357

Paul W. E., Seder R. A. 1994. Lymphocyte responses and cytokines. *Cell* 76:241

Pene J., Rousset F., Briere F., et al. 1988. IgE production by normal human lymphocytes is induced by interleukin 4 and suppressed by interferons γ and α and prostaglandin E_2. *Proc. Natl. Acad. Sci. USA* 85:6880

Pene J., Chretien I., Rousset F., Briere F., Bonnefoy J. Y., de Vries J. 1989. Modulation of IL-4-induced human IgE production *in vitro* by IFNγ and IL-5: the role of soluble CD23 (sCD23). *J. Cell. Biochem.* 39:253

Peng Z., Lee H.-B., Proud D., Naclerio R., Adkinson N. F. JR 1990. Ragweed IgE and IgG_4 antibody in nasal secretions during immunotherapy. *Clin. Exp. Allergy* 20:571

Peng, Z., Naclerio, R. M., Norman, P. S., Adkinson, N. F. 1992. Quantitative IgE- and IgG-subclass responses during and after long-term ragweed immunotherapy. *J. Allergy Clin. Immunol.* 89:519

Pernis A., Gupta S., Gollob K. J., Garfein E., Coffman R. L., Schindler C., Rothman P. 1995. Lack of interferon γ receptor β chain and the prevention of interferon γ signalling in Th1 cells. *Science* 269:245

Punnonen J., de Vries J. E. 1994. IL-13 induces proliferation, Ig isotype switching, and Ig synthesis by immature human fetal B cells. *J. Immunol.* 152:1094

Punnonen J., Aversa G., Cocks B. G., McKenzie A. N. J., Menon S., Zurawski G., de Waal Malefyt R., de Vries J. E. 1993. Interleukin-13 induces interleukin-4-independent IgG4 and IgE synthesis and CD23 expression by human B cells. *Proc. Natl. Acad. Sci. USA* 90:3730

Punnonen J., Punnonen K., Jansén C. T., Kalimo K. 1993. Interferon (IFN)-α, IFN-γ, interleukin (IL)-2, and arachidonic acid metabolites modulate IL-4-induced IgE synthesis similarly in healthy persons and in atopic dermatitis patients. *Allergy* 48:189

Reiner S. L., Seder R. A. 1995. T helper cell differentiation in immune response. *Curr. Opin. Immunol.* 7:360

Romagnani S. 1991. Human Th1 and Th2: Doubt no more. *Immunol. Today* 12:256

Schneider T., Dudler T., Gelb M. H., Suter M. 1994. Lysine residues in bee venom phospholipase A_2 are important for binding to human monoclonal or polyclonal antibodies of the IgG4 isotype. *Int. Arch. Allergy Immunol.* 104:262

Schroeder J. T., MacGlasham D. W. Jr., Kagey-Sobotka A., White J. M., Lichtenstein L. W. 1994. IgE-dependent IL-4 secretion by human basophils. *J. Immunol.* 153:1808

Secrist H., DeKruyff R. H., Umetsu D. T. 1995. Interleukin-4 production by $CD4^+$T cells from allergic individuals is modulated by antigen concentration and antigen-presenting cell type. *J. Exp. Med.* 181:1081

Seder R. A., Paul W. E., Davis M. M., Fazekas de St. Groth B. 1992. The presence of interleukin-4 during in vitro priming determines the lymphokine-producing potential of $CD4^+$T cells from T cell receptor transgenic mice. *J. Exp. Med.* 176:1091

Seder R. A., Gazzinelli R., Sher A., Paul W. E. 1993. IL-12 acts directly on $CD4^+$T cells to enhance priming for IFNγ production and diminishes IL-4 inhibition of such priming. *Proc. Natl. Acad. Sci. USA* 90:10188

Seder R. A., Grabstein K. H., Berzofsky J. A., and McDyer J. F. 1995. Cytokine interactions in human immunodeficiency virus-infected individuals: roles of interleukin (IL)-2, IL-12, and IL-15. *J. Exp. Med.* 182:1067

Sutherland M., Blaser K., Pène J. 1993. Effects of interleukin-4 and interferon-γ on the secretion of IgG4 from human peripheral blood mononuclear cells. *Allergy* 48:504

Swain S. L. 1994. Generation and *in vivo* persistence of polarized Th1 and Th2 memory cells. *Immunity* 1:543

Tagaya Y., Bamford R. N., DeFilippis A. P., Waldmann T. A. 1996. IL-15: A pleiotropic cytokine with diverse receptor/signalling pathways whose expression is 7controlled at multiple levels. *Immunity* 4:329

Tang M. L. K., Coleman J., Kemp A. S. 1995. Interleukin-4 and interferon-gamma production in atopic and non-atopic children with asthma. *Clin. Exp. Allergy* 25:515
Tang M., Kemp A., Varigos G. 1993. IL-4 and interferon-gamma production in children with atopic disease. *Clin. Exp. Immunol.* 92:120

Tikkainen U., Klockars M. 1989. Determination of IgG subclass antibodies against wheat flour antigens by an ELISA technique. *Allergy* 44:419

Tsai L.C., Tang R.-B., Hung M.-W., Chang Z.-N. 1990. Changes in the levels of house dust mite specific IgG_4 during immunotherapy in asthmatic children. *Clin. Exp. Allergy* 21:367

Van Der Pouw Kraan C. T. M., Aalberse R. C., Aarden L. A. 1994. IgE production in atopic patients is not related to IL-4 production. *Clin. Exp. Immunol.* 97:254

Wallner B. P., Gefter M. L. 1994. Immunotherapy with T cell reactive peptides derived from allergens. *Allergy* 49:302

Wilkinson P. C., Liew F. Y. 1995. Chemoattraction of human blood T lymphocytes by interleukin-15. *J. Exp. Med.* 181:1255

Yang L.-P., Demeure C. E., Byun D.-G., Vezzio N. 1995. Maturation of neonatal human CD4 T cells III. Role of B7 co-stimulation at priming. *Int. Immunol.* 7:1987

Yang X., HayGlass K. T. 1993. IFNγ, but not IL-4, synthesis by antigen-primed murine T cells is IL-2 dependent. *J. Immunol.* 150:4354

Young R. P., Dekker J. W., Wordsworth B. P., Schou C., Pile K. D., Matthiessen F., Rosenberg W. M. C., Bell J. I., Hopkin J. M., Cookson W. O. C. M. 1994. HLA-DR and HLA-DP genotypes and immunoglobulin E responses to common major allergens. *Clin. Exp. Allergy* 24:431